Characterizing the localization and role of \textit{lin-5} and \textit{era-1} mRNAs in early \textit{C. elegans} embryos

THÈSE N° 6355 (2014)
PRÉSENTÉE LE 26 SEPTEMBRE 2014
À LA FACULTÉ DES SCIENCES DE LA VIE
UNITÉ DU PROF. GÓNCZY
PROGRAMME DOCTORAL EN APPROCHES MOLÉCULAIRES DU VIVANT

ÉCOLE POLYTECHNIQUE FÉDÉRALE DE LAUSANNE
POUR L’OBTENTION DU GRADE DE DOCTEUR ÉS SCIENCES

PAR

Zoltán Péter SPIRÓ

acceptée sur proposition du jury:
Prof. J. Lingner, président du jury
Prof. P. Gónczy, directeur de thèse
Prof. D. Constam, rapporteur
Dr A. Ephrussi, rapporteuse
Dr H. Grosshans, rapporteur
Nagymamának
Summary

Asymmetric cell division is essential for the generation of diversity during development and the function of stem cell lineages. The *Caenorhabditis elegans* zygote is an attractive model to investigate the mechanisms of spindle positioning during asymmetric cell division. In this polarized cell, the asymmetric distribution of cortical force generators along the antero-posterior axis and pulling on astral microtubules leads to the unequal cleavage of the one-cell embryo. The mechanisms underlying such cortical force generation are thought to act strictly at the protein level.

In this thesis work we report that the mRNA encoding the cortical force generator component LIN-5 is enriched around centrosomes in early embryos, in a manner that depends on microtubules and dynein. We found a likewise enrichment in *C. briggsae*, indicating evolutionary conservation of such localization. We established that the lin-5 coding sequence is necessary and sufficient for mRNA enrichment around centrosomes in *C. elegans*. In addition, we found that lin-5 mRNA is mislocalized in lin-5(*ev571*) mutant embryos, which harbor a 9 nucleotide insertion in the coding sequence. Moreover, an intragenic revertant of lin-5(*ev571*), lin-5(*ev571he63*), also exhibits mislocalized lin-5 mRNA distribution. We demonstrated that this is accompanied by diminished pulling forces on the posterior spindle pole, suggesting that centrosomal localization of lin-5 mRNA is important for robust pulling forces. We found also that lin-5 mRNA centrosomal enrichment is slightly asymmetric during anaphase, with more transcripts present on the anterior side. We developed a novel FRAP-based assay, which revealed that lin-5 is translated/folded preferentially in the cytoplasm compared to centrosomes. Furthermore, we found that morpholino-mediated inhibition of lin-5 translation diminishes pulling forces on the posterior side during anaphase. Together, these findings lead us to propose that preferential translation/folding of lin-5 in the posterior cytoplasm following release of the mRNA from the posterior centrosome contributes to asymmetric cortical distribution of force generators, and thus to proper spindle positioning.

Moreover, we found that the mRNA of an uncharacterized gene, era-1 is enriched on the anterior side of the zygote and is inherited by the anterior blastomeres. Similar to era-1 mRNA, a YFP fusion of ERA-1 protein is also
asymmetrically distributed. *era-1* mRNA and protein asymmetry is disturbed when the polarity of the cell is compromised. Moreover, asymmetric distribution of both *era-1* mRNA and YFP-ERA-1 protein requires the *era-1* 3′ UTR. Furthermore, the RNA-binding protein MEX-5 is needed for both asymmetric *era-1* mRNA localization and for its translational activation.

Furthermore, we report that the clathrin heavy chain CHC-1 negatively regulates pulling forces acting on centrosomes during interphase and on spindle poles during mitosis in one-cell *C. elegans* embryos. We establish a similar role for the cytokinesis/apoptosis/RNA-binding protein CAR-1 and uncover that CAR-1 is needed to maintain normal levels of CHC-1. We demonstrate that CHC-1 is necessary for proper organization of the cortical acto-myosin network and for full cortical tension. Furthermore, we establish that the centrosome positioning phenotype of embryos depleted of CHC-1 is alleviated by stabilizing the acto-myosin network. Conversely, we demonstrate that slight perturbations of the acto-myosin network in otherwise wild type embryos results in excess centrosome movements resembling those in *chc-1(RNAi)* embryos. Overall, our findings lead us to propose that clathrin plays a critical role in centrosome positioning by promoting acto-myosin cortical tension.

**Keywords**: *C. elegans* embryos, asymmetric cell division, pulling forces, *lin-5* mRNA, *era-1* mRNA, clathrin heavy chain
**Résumé**

La division cellulaire asymétrique est essentielle pour assurer la diversité au cours du développement et le fonctionnement de lignées de cellules souches. Le zygote de *Caenorhabditis elegans* est un modèle intéressant pour étudier les mécanismes de positionnement du fuseau mitotique lors de la division cellulaire asymétrique. Dans cette cellule polarisée, la répartition asymétrique des générateurs de force corticaux le long de l'axe antéro-postérieur et tirant sur les microtubules astraux conduit à un clivage inégal du zygote. Les mécanismes à l'origine de la génération d'une telle force corticale sont censés agir strictement au niveau des protéines.

Dans ce travail de thèse, nous rapportons que l'ARNm qui code le composant générateur de force corticale LIN-5 est enrichi autour des centrosomes dans les embryons précoces, d'une manière qui dépend des microtubules et de la dynéine. Nous avons également observé un enrichissement chez *C. briggsae* qui indique une conservation de cette localization au cours de l'évolution. Nous avons établi que la séquence codante de *lin-5* est nécessaire et suffisante pour l'enrichissement d'ARNm autour des centrosomes chez *C. elegans*. En outre, nous avons constaté que le *lin-5* ARNm est localisé de manière uniforme chez les *lin-5(ev571)* embryons mutants qui ont une insertion des neuf nucléotides dans la séquence codante. Un révertant intragénique de *lin-5(ev571), lin-5(ev571he63)*, manifeste également une distribution de *lin-5* ARNm uniforme. Nous avons démontré que ceci s'accompagne d'une diminution des forces de traction sur le pôle postérieur du fuseau mitotique, ce qui suggère que la localisation centrosomale de *lin-5* ARNm est importante pour obtenir des forces de traction robustes. Nous avons également constaté que l'enrichissement centrosomal des *lin-5* ARNm est légèrement asymétrique lors de l'anaphase, avec plus de transcripts présents sur du côté antérieur. Nous avons développé un nouveau test sur la base du FRAP, qui a révélé que le *lin-5* ARNm est traduit / plié de manière préférentielle dans le cytoplasme par rapport à centrosomes. En outre, nous avons trouvé que l'inhibition induite par morpholino de la traduction de *lin-5* diminue les forces de traction du côté postérieur lors de l'anaphase. Ensemble, ces résultats nous
permettent de proposer que la traduction / pliage préférentiels de lin-5 dans le cytoplasme postérieur après la libération de l'ARNm du centrosome postérieur contribuent à la distribution corticale asymétrique de générateurs de force, et donc au positionnement approprié du fuseau mitotique.

En outre, nous avons constaté que l'ARNm d'un gène non caractérisé, era-1, est enrichi du côté antérieur du zygote et est hérité par les blastomères antérieurs. D'une façon similaire au era-1 ARNm, une fusion YFP de la protéine ERA-1 est également distribuée de manière asymétrique. L'asymétrie de l'ARNm de era-1 ainsi que de YFP-ERA-1 sont perturbées quand la polarité de la cellule est compromise. La distribution asymétrique de era-1 ARNm et YFP-ERA-1 nécessite l'era-1 3'UTR. En outre, la protéine de liaison à l'ARN MEX-5 est nécessaire à la fois pour la localisation et l'activation traductionnelle de l'era-1 asymétrique.

Finalement, nous rapportons que la chaîne lourde de la clathrine CHC-1 régule négativement les forces de traction agissant sur les centrosomes pendant l'interphase et sur les poles du fuseau lors de la mitose dans le zygote de C. elegans. Nous établissons un rôle similaire pour la cytokinèse/apoptose/protéine de liaison à l'ARN CAR-1 et observons que la CAR-1 est nécessaire pour maintenir des niveaux normaux de CHC-1. Nous démontrons que CHC-1 est nécessaire pour la bonne organisation du réseau acto-myosine cortical et pour une tension corticale complète. En outre, nous établissons que le phénotype de positionnement du centrosome des embryons déplétés de CHC-1 par RNAi est atténué par la stabilisation du réseau acto-myosine. Inversément, nous démontrons que de légères perturbations du réseau acto-myosine dans des embryons de type sauvage provoquent un excès de mouvements des centrosomes qui ressemble à ceux des embryons chc-1(RNAi). Dans l'ensemble, nos résultats nous amènent à proposer que la clathrine joue un rôle essentiel dans le positionnement du centrosome par la promotion de la tension corticale du réseau acto-myosine.

Mots-clés: embryons de C. elegans, division cellulaire asymétrique, forces de traction, lin-5 ARNm, era-1 ARNm, chaîne lourde de la clathrine
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1. INTRODUCTION

1A. Asymmetric cell division

The generation of various cell types is a prerequisite for the establishment of a multicellular organism. A fundamental mechanism by which such diversity of cell types is achieved during development is asymmetric cell division (reviewed in Gönczy, 2008). The resulting two daughter cells following an asymmetric division acquire different fates and give rise to distinct tissues in the adult organism. Asymmetric cell division is present in unicellular organisms as well as in plants and animals, and is also crucial for the maintenance of stem cell lineages (Figure 1 and reviewed in Knoblich, 2008).

Figure 1. Asymmetric cell division in various organisms.
The position of the mitotic spindle determines the division plane in animal cells. Therefore spindle positioning is important in determining if a given cell division is symmetric or asymmetric (compare Figure 2A with C). There are several types of asymmetric cell division. In the case of extrinsic asymmetric cell divisions, the two daughter cells are identical at the time of birth and subsequent signaling stemming from the neighbors drives the distinction between them. By contrast, a cell undergoing intrinsic asymmetric cell division possesses polarized domains prior to division, leading to the differential segregation of various cell fate determinants (proteins and/or RNAs) and organelles in the newborn cells. Moreover, depending on the relative sizes of the two daughter cells, an intrinsically determined asymmetric cell division can be equal or unequal, a feature depending on the position of the mitotic spindle (compare Figure 2C with D).

Figure 2. Types of cell divisions
A-D Depending on the plane of division, cell fate determinants (blue triangle, red square and yellow octagon) are segregated differently in the daughter cells, leading to various cell fates (colored cells in the third row). Equal symmetric (A), equal extrinsic asymmetric (B), equal intrinsic asymmetric (C) and unequal intrinsic asymmetric (D) cell divisions are depicted. The plane of division is shown as a purple dotted line in the first column.
Axis formation during *C. elegans* development

The early development of *C. elegans* provides an excellent example of how asymmetric segregation of cell fate determinants executed by asymmetric cell divisions is capable of setting up the three main body axes within 4 rounds of division (Figure 3). Through these divisions, six founder cells (AB, MS, E, C, D and P₄) are generated that are responsible for building up the various tissues of the embryo (Sulston et al., 1983). The antero-posterior (A-P) axis is defined shortly after fertilization in the zygote (see below for details). This cell, called P₀, divides asymmetrically along the A-P axis, giving rise to a larger anterior cell, named AB, and a smaller posterior cell, P₁ (Figure 3A and B). Thereafter, AB divides symmetrically generating an anteriorly and a posteriorly positioned cell, ABₐ and ABₚ, respectively, whereas P₁ divides asymmetrically, leading to the formation of the larger EMS and the smaller P₂ cells (Figure 3C). The dorso-ventral axis is set up at this stage, as the position of the midbody remnant arising from the previous cell division drive the position of EMS, thus establishing the ventral side of the embryo (Singh and Pohl, 2014). The left-right axis is created by the symmetric divisions of ABₐ and ABₚ that generate ABₐ₁ and ABₚ₁, which define the left side of the embryo. On the opposite side, ABₐr and ABₚr mark the presumptive right side. In this third round of division, EMS and P₂ divide again asymmetrically, generating MS and E, as well as C and P₃ respectively (Figure 3D). Thereafter the asymmetric division of P₃ gives rise to D and P₄ (Figure 3E). The descendants of each founder cell differentiate into specific cell types later on as follows: AB - hypodermis, neurons, pharynx; MS – muscle, pharynx, neurons, somatic gonad; C- muscle, hypodermis, neurons; D – muscle; E – intestine, - P₄ - germ line (Figure 3F). Of note, the nomenclature of some of the genes reflects defects in cell specification when mutated or upon RNAi-mediated depletion. Some examples for such genes will be given in the later sections.
Figure 3. Early embryonic development of *C. elegans*

A-E The first four rounds of cell division give rise to the major three embryonic axes and to the generation of six founder cells. 1-cell stage (A), 2-cell stage (B), 4-cell stage (C), 16-cell stage (D), 64-cell stage (E). The name of the cells is indicated; in panels D and E, only the first layer of cells is seen. *F* Generation of the six founder cells (AB, MS, E, C, D, P) through the first four cell divisions. The tissues generated from each founder cell are listed below the cell.

Model systems to study asymmetric cell division

The *C. elegans* zygote

Given its large size (50x30µm), its relative ease of handling and the available genetic and genomic tools, the first cell division of the *C. elegans* zygote became an attractive model system to study asymmetric cell division. The phenotypic events occurring in this cell are easily detectable using Differential Interference Contrast (DIC) microscopy (Figure 4A; Albertson, 1984; Nigon et al., 1960; Gönczy et al., 1999). Furthermore, an increasing number of fluorescently-tagged transgenic lines
allow researchers to detect proteins and analyze their dynamics with high spatial and temporal resolution, as is exemplified in embryos shown in Figure 4B-E. Moreover, in situ hybridization and immunofluorescence methods have been optimized to reveal localization of mRNAs and proteins in fixed specimens. In the following sections, the events occurring in the one-cell stage embryo as well as the molecular mechanisms leading to its asymmetric division are introduced.

**Processes in the one-cell stage embryo**

The sperm, containing the paternal DNA and a pair of centrioles, fertilizes the oocyte. Fertilization triggers the completion of female meiosis I and, shortly thereafter, the two pronuclei appear on the two sides of the zygote (pronuclear formation, Figure 4A). The female pronucleus is located at the presumptive anterior, the male at the presumptive posterior side of the embryo. While the two pronuclei increase in size, the plasma membrane ingresses at the embryo center from both sides, mimicking cytokinesis. This ingression is the pseudocleavage furrow. Then, both the female and the male pronuclei migrate towards the center of the cell with the female pronucleus moving faster; therefore pronuclear meeting occurs at the posterior side. The two pronuclei are now associated with the centrosomes that are aligned orthogonal to the A-P axis. This nucleo-centrosomal complex moves toward the center of the embryo while undergoing a 90° rotation (this process is named centration/rotation). At the end of this process, the two pronuclei and the associated centrosomes are located in the middle of the cell, aligned parallel to the A-P axis. Following nuclear envelope breakdown (NEBD), the mitotic spindle is formed in the cell center. During metaphase, the spindle is displaced towards the posterior side of the cell and this displacement becomes even more pronounced in anaphase. During anaphase, the posterior spindle pole undergoes oscillations perpendicular to the A-P axis, which dampen at the onset of cytokinesis. The cytokinetic furrow ingresses and bisects the mitotic spindle. As a result, the zygote is split into two daughter cells with different sizes, a larger anterior AB blastomere and a smaller posterior P₁ blastomere. Still images of a zygote recorded by time-lapse DIC microscopy are shown in Figure 4A.
Molecular mechanisms

An intrinsic asymmetric cell division can be thought of as occurring through four steps (Figure 4). First, the initial symmetry of the cell must be broken. Second, polarization leads to distinct domains within the cell. Third, cell fate determinants segregate in response to this polarization. Fourth, the mitotic spindle is oriented in a way that ensures the proper segregation of cell fate determinants.

Figure 4. The first cell division of the *C. elegans* embryo
Four steps of an asymmetric division (Symmetry breaking, Polarity establishment, Fate determinant segregation and Spindle positioning) are shown in embryos expressing different fusion proteins. The embryo and stage where one of the four steps of asymmetric cell division discussed in the text is visible is marked with a colored rectangle. A Snapshots from Differential Interference Contrast (DIC) recording visualizing the first cell division of a GFP-Histone2B;GFP-α-tubulin expressing embryo. The stages are indicated on top of the images. The male and the female pronuclei are indicated in the second frame. In all the embryos shown in this thesis, anterior is to the left, posterior to the right. Here and elsewhere, unless otherwise stated, scale bar represents 10μm, t=0 corresponds to cytokinesis onset. B The GFP channel visualizes the DNA and the microtubules in the embryo shown in A. Cell cycle stages are indicated above each frame. Two arrows mark the centrosomes in the second frame. C Embryo expressing a GFP fusion of the non-muscle myosin II (NMY-2) (Image source: Munro et al., 2004). The stages of the embryo here and in panel D and E roughly correspond.

Symmetry breaking

Following fertilization, the acto-myosin rich meshwork located just below the oocyte cell membrane (hereafter referred to as the cell cortex) undergoes contractions throughout the whole cell. These contractions are based on the activity of the non-muscle myosin II, NMY-2 and involve also the Rho family GTPase RHO-1, as well as the Rho binding kinase LET-502 (Kumfer et al., 2010; Motegi and Sugimoto, 2006; Munro et al., 2004; Schonegg and Hyman, 2006; Shelton et al., 1999). Upon fertilization, these cortical contractions cease locally in the vicinity of the male pronucleus (Bienkowska and Cowan, 2012; Cheeks et al., 2004; Cowan and Hyman, 2004a; Cuenca et al., 2003; Hamill et al., 2002; Munro et al., 2004). Why are these contractions locally inactivated? The answer lies in the fact that the centrosome brought in by the sperm triggers symmetry breaking. Indeed, laser ablation of centrosomes (Cowan and Hyman, 2004b; Hamill et al., 2002) or interfering with centrosomal proteins, like SPD-2, SPD-5 or CYE-1 (Cowan and Hyman, 2006; Hamill et al., 2002; O’Connell et al., 2000) leads to the absence of symmetry breaking. At the molecular level, the RHO-1 G-protein exchange factor (GEF) ECT-2, previously present all over the cell periphery, is locally removed from the cortex near the centrosomes (Motegi and Sugimoto, 2006; Schonegg and Hyman, 2006). On the other hand, CYK-4, a RHO-1 GAP introduced by the sperm is thought to be also important in this process, as removal of CYK-4 from sperm cells compromises symmetry breaking (Jenkins et al., 2006). The involvement of microtubules in this process is somewhat controversial, given that polarity was reported to be unaffected in the absence of α-tubulin tba-2 and γ-tubulin tbg-1 (Cowan and Hyman, 2004b; Sonneville and Gönczy, 2004), but a significant delay in polarity establishment has been observed in zygotes expressing GFP-tagged PAR-6 lacking both α- and β-tubulin (Tsai and Ahringer, 2007). This controversy has recently been solved by Motegi and coworkers (Motegi et al., 2011), who showed that the microtubule-mediated loading of the posterior polarity protein PAR-2 is compromised in the
absence of microtubules, leading to delay in polarization that becomes corrected afterwards.

**Polarity establishment and maintenance**

After symmetry breaking, the smooth, non-contractile cortical region retracts towards the presumptive anterior and reaches ~50% egg-length (Figure 4C; Cuenca et al., 2003; Hird and White, 1993). This process is concomitant with an anteriorly-directed cortical flow initiating from the future posterior side. This contraction of the acto-myosin towards the anterior results in a sequence of events that establishes the anterior and the posterior domains of the cell. Six *partitioning defective* (*par*) are key in building these two domains: the anterior domain comprise the PDZ proteins PAR-3 and PAR-6 and the atypical protein kinase PKC-3, while the posterior domain consists of the Ser/Thr kinase PAR-1 and the RING-finger protein PAR-2. Moreover, the Ser/Thr kinase PAR-4 and the 14-3-3 protein PAR-5 are also crucial for proper polarity. The anterior PAR-complex, initially enriched all over at the cell cortex (Etemad-Moghadam et al., 1995; Hung and Kemphues, 1999; Tabuse et al., 1998) becomes cleared from the posterior and retracts together with the acto-myosin cortex as judged by the concomitant movements of GFP-NMY-2 and GFP-PAR-6 (Cuenca et al., 2003). In turn, PAR-2 and PAR-1 are recruited to these cleared posterior cortical regions (Figure 4D; Boyd et al., 1996; Guo and Kemphues, 1995). Although the exact mechanisms of this recruitment is unclear, it has been shown that PAR-2 is a microtubule- and lipid-binding protein that can localize to the cortex and lead to proper polarization even in the absence of cortical flows (Motegi et al., 2011; Zonies et al., 2010). The acto-myosin network and the PAR proteins are interdependent for polarization, as actin is needed for polarization (Guo and Kemphues, 1996; Hill and Strome, 1988; Shelton et al., 1999) and polarization of NMY-2 is abolished upon depletion of *par-3*, *par-4*, *par-5* or *par-6* (Cheeks et al., 2004; Cuenca et al., 2003; Morton et al., 1992; Munro et al., 2004). What is the mechanical driving force for the anterior-directed cortical movements of the acto-myosin network? Anisotropies in cortical tension is the answer (Mayer et al., 2010). Cortical tension, measured by outward velocities of GFP-NMY-2 foci following a cortical laser cut (cortical laser ablation, COLA) parallel to the A-P axis is uniform throughout the embryo. By contrast, the tension orthogonal to the A-P axis is twice as high in the anterior cortex.
as compared to the posterior one. This difference results in anisotropies that can serve as mechanical basis for cortical flows (Mayer et al., 2010). As a result of this dynamic polarization process, the *C. elegans* zygote has two distinct anterior and posterior cortical domains at the stage of pseudocleavage (Figure 4D). The maintenance of these two domains is executed through mutual inhibition between the anterior and posterior domain. First, PKC-3 phosphorylates PAR-2, thereby preventing PAR-2 from localizing to the anterior cortex (Hao et al., 2006). In turn, PAR-2, in concert with PAR-1 and PAR-5, inhibits the posterior localization of the anterior PAR complex (Hao et al., 2006). The small GTPase CDC-42 contributes to polarity maintenance by remodeling the actin cytoskeleton. Moreover, CDC-42*GTP has been shown to interact and colocalize with PAR-6 (Aceto et al., 2006; Gotta et al., 2001; Schonegg and Hyman, 2006). Furthermore, CDC-37, a member of the Hsp90 family stabilizes PKC-3 thereby helping maintaining polarity (Beers and Kemphues, 2006). Apart from these proteins, there are other associated components involved in polarity establishment and maintenance that are not discussed here (reviewed in Gönczy and Rose, 2005).

### Segregation of cell fate determinants

The newly established cortical asymmetry must result in cytoplasmic asymmetries of cell fate determinants along the antero-posterior (A-P) axis in order to achieve proper developmental patterning. A few asymmetries are already present at the end of the first cell cycle, such as the distribution of MEX-5/6, PIE-1 and P-granules. Other polarity mediators and cell fate regulators become enriched in distinct cells later on during development; some of these proteins will be mentioned in the following sections.

MEX-5 and MEX-6 (from *muscle excess*, hereafter referred to collectively as MEX-5/6) are two highly identical and partially redundant CCCH zinc finger proteins (Schubert et al., 2000). MEX-5/6 distribution show a polarity-dependent antero-posterior gradient at the time of pronuclear meeting, with higher levels in the anterior (Figure 4E; Cuenca et al., 2003; Schubert et al., 2000). Interestingly, higher mobility of MEX-5 in the posterior has been suggested to drive this gradient (Daniels et al., 2010; Tenlen et al., 2008). PAR-1 contributes to the establishment of differential
MEX-5 mobility by phosphorylating it (Griffin et al., 2011). This leads to the release of MEX-5 from RNA-binding complexes, thereby increasing its mobility specifically in the posterior side. In the anterior, reduced level of PAR-1 is overcome by dephosphorylation executed by PP2A phosphatase, leading to lower MEX-5 mobility at the anterior (Griffin et al., 2011). Furthermore, a positive feedback loop ensures proper polarity establishment. MEX-5/6 themselves promote the posterior expansion of PAR-2 and PAR-1, presumably by clearing the anterior PARs from the posterior (Cuenca et al., 2003), but the exact molecular mechanism remains elusive. MEX-5/6 are crucial for setting up soma-germline asymmetries by regulating the distribution of numerous proteins. In embryos depleted of mex-5 and mex-6, the posterior enrichment of various cell fate determinants, like PIE-1, SKN-1, MEX-1, POS-1 and PAL-1 becomes symmetric (Schubert et al., 2000). Moreover, in the 4-cell stage embryo, MEX-5/6 contributes to the anterior enrichment of the Notch receptor GLP-1 (Schubert et al., 2000).

PIE-1 (pharynx and intestine in excess) is another CCCH zinc finger containing protein whose distribution in P₀ is the converse of MEX-5/6 (Cuenca et al., 2003; Mello et al., 1996; Reese et al., 2000): PIE-1-GFP shows posterior enrichment at the time of pronuclear meeting and is inherited in the posterior blastomeres in a polarity-dependent manner (Reese et al., 2000). Interestingly, like in the case of MEX-5/6, differential diffusion coefficients between the anterior and posterior sides are responsible for setting up the PIE-1 gradient (Daniels et al., 2010). The initial asymmetry in PIE-1 levels is further reinforced by MEX-5/6-regulated proteasomal degradation of residual PIE-1 in somatic cells (DeRenzo et al., 2003). PIE-1 is crucial for germ line development by inhibiting transcription of soma-related genes in the P-lineage and by promoting translation of maternally provided genes such as nos-2 and apx-1 (Mickey et al., 1996; Seydoux and Dunn, 1997; Seydoux et al., 1996; Tenenhaus et al., 2001).

P-granules are protein-RNA complexes that are important for germline specification and are analogous to the perinuclear nuage in mouse and human germ cells (reviewed in Updike and Strome, 2010). P-granule assembly in the germline is disrupted in deps-1, glh-1, pgl-1, and ife-1 mutants (Amiri et al., 2001; Kawasaki et al., 1998; Spike et al., 2008a; Spike et al., 2008b) and simultaneous mutations in these four genes results in germ line underproliferation and failure in oocyte and sperm
production (Amiri et al., 2001; Kawasaki et al., 2004; Spike et al., 2008a; Spike et al., 2008b). In the early embryos, P-granules dynamically localize to the posterior part of \( P_0 \) during centration/rotation and thus they are inherited by \( P_1 \) (Figure 1C and Figure 62A; Strome and Wood, 1983). Subsequently, P-granules are inherited again strictly by the P-lineage during later development. The asymmetric distribution of P-granules is under the control of A-P polarity cues (Guo and Kemphues, 1995; Tabuse et al., 1998) and immunostaining with antibodies directed against the P-granule component PGL-1 has been widely used as marker of proper polarity in the one-cell stage. Surprisingly, asymmetric distribution of P-granules is dispensible for germline development, as embryos depleted of the PP2A phosphatase regulatory subunit \( pptr-1 \) exhibit homogenous P-granule distribution whilst the majority of the resulting worms undergo apparently normal germline development (Gallo et al., 2010). This suggests that asymmetric P-granule distribution is rather a consequence of, and not the driving force for, germline specification.

**Spindle positioning**

An asymmetrically dividing cell must position its spindle in a way that cell fate determinants are inherited properly by the daughter cells after cell division. As mentioned before, the first cell division of the *C. elegans* zygote is unequal, giving rise to a larger anterior and a smaller posterior cell. Spindle positioning in this zygote includes two steps: spindle orientation and spindle displacement. Centration/rotation ensures that the two centrosomes are located in the center of the embryo and aligned parallel to the A-P axis. Thus, the newly formed spindle is set up in the center with a proper orientation. Afterwards, the spindle is displaced towards the posterior part of the cell, giving rise to the characteristic excentric spindle position and unequal cleavage plane (Figure 4A).

**ACTIN AND MICROTUBULES**

Cytoskeletal components, namely the actin network as well as microtubules are involved in the mechanisms driving centrosome and spindle positioning.

The actin network is one of the three main types of biopolymers building up the cytoskeleton. Actin filaments (F-actin) are polarized flexible structures with a
diameter of 5-9 nm composed of two parallel protofilaments twisting around each other (reviewed in Alberts et al., 2002, chapter 16). Actin filaments are most abundant in the cell periphery, thereby forming a structure called the cell cortex. Actin filaments are very dynamic and this dynamicity is regulated by several actin-associated proteins (reviewed in Allard and Mogilner, 2013; Blanchoin et al., 2014). Non-muscle myosin II is a molecular motor that binds to actin filaments and is able to move towards the plus end of the actin filament utilizing ATP hydrolysis. Major functions of F-actin include cell shape maintenance, contraction, motility and cytokinesis.

Microtubules are composed of parallel protofilaments joined laterally, thereby building up a hollow cylinder with a diameter of 25 nm (reviewed in Alberts et al., 2002, chapter 16). The protofilaments comprise globular subunits of α-tubulin and β-tubulin heterodimers. Microtubules are polarized structures possessing a more dynamic plus-end, where the heterodimers can be incorporated in the structure upon GTP-hydrolysis of β-tubulin. Microtubule nucleation occurs at the microtubule organizing centers (MTOCs) that contains the γ-tubulin ring complex (reviewed in Kollman et al., 2011). In animal cells, the main MTOC is the centrosome. Microtubules constantly undergo growth and shrinkage, leading to their dynamic instability. Plus (kinesins) and minus (dynein) end directed motor protein complexes are able to move along microtubules by hydrolyzing ATP, thereby contributing to a variety of cellular processes, such as organelle transport, nuclear migration and spindle positioning (reviewed in Roberts et al., 2013).

Forces driving centrosome and spindle positioning can be actin and/or microtubule-dependent. Thus, interfering with actin dynamics blocks meiotic spindle positioning in mouse oocytes (Li et al., 2008; Schuh and Ellenberg, 2008). By contrast, actin depolymerization does not prevent spindle positioning in C. elegans zygotes (Hill and Strome, 1988). However, dynamic microtubules are crucial, as treating mitotic embryos with the microtubule depolymerizing agent nocodazole leads to exaggerated spindle displacement (Gönczy et al., 2001). Conversely, stabilizing microtubules with taxol or using a temperature sensitive allele of the β-tubulin gene tbb-1 result in more stable microtubules interferes with unequal cell division (Nguyen-Ngoc et al., 2007).
FORCES DRIVING CENTRATION/ROTATION

Nuclear positioning to the center in animal cells can be exerted in several ways (Figure 5). This includes pushing force arising from microtubule polymerization, whereby newly incorporated tubulin dimers exert force on the microtubule (Figure 5A). Minus-end directed motor proteins such as dynein are also able to move nuclei in several ways, depending on the place of anchoring. These motors can be anchored in the cytoplasm (Figure 5B), on the cortex (Figure 5C) or on the nucleus (Figure 5D) resulting in force generation and nuclear movements (reviewed in Reinsch and Gönczy, 1998). Motor proteins anchored in the cytoplasm give rise to length-dependent force, as the longer the microtubule is, the more motor proteins can bind and the more force can be generated.

**Figure 5. Different mechanisms of nuclear positioning.**

Nuclear positioning can be executed by pushing force arising from microtubule polymerization (A), by minus-end directed motor proteins anchored in the cytoplasm (B) or else on the cortex (C), or by motor proteins bound to the nucleus (D). The nucleus (blue circle), the MTOC (purple circle), tubulin dimers (green circles) forming a microtubule and minus-end directed motors (red triangle) are shown. The lower image displays the resulting movement of the nucleus. The polarity of microtubules is indicated with – and + symbols. Individual motors exert force (red arrows) that result in a net force (orange arrow). Newly incorporated tubulin dimers in A are shown in bright green.

In *C. elegans*, after pronuclear meeting, the two pronuclei and the centrosomes move to the cell center. What is the nature of the forces driving this movement? At an earlier stage, computer simulation revealed that, during female pronuclear migration, forces act in a length dependent manner (Kimura and Onami, 2005). It has been
postulated that similar mechanisms act during centration/rotation and that force generator entities are bound to endomembranes distributed throughout the cell (Kimura and Kimura, 2011). In this model, endomembrane movements towards the minus-end of microtubules generate an overall pulling force on the centrosome acting in the opposite direction. Depletion of DYRB-1, a light chain subunit of cytoplasmic dynein, blocks centration as well as the movements of early and late endosomes. Interfering with the early and late endosomal proteins RAB-5 or RAB-7 slows down the pace of centration without blocking it completely (Kimura and Kimura, 2011), indicating that this regulation is likely only part of the whole picture.

FORCES DRIVING SPINDLE DISPLACEMENT

Where are the forces driving spindle displacement generated? Microtubule-dependent pushing force intrinsic to the spindle (Leslie and Pickett-Heaps, 1983) or forces external to the spindle and pulling on astral microtubules (Aist and Berns, 1981; Aist et al., 1993; Berns et al., 1981) have been implicated in spindle pole separation in other systems. In the case of the *C. elegans* zygote, the latter seems to be the major source of force generation. This conclusion was drawn from experiments in which the anaphase spindle was severed by a UV laser; as a consequence, the liberated spindle poles exhibited outward movements (Figure 6; Grill et al., 2001). Moreover, centrosomal fragments following optically induced centrosomal disintegration (OICD) travelled all the way until they reached the cell periphery, indicative of cortically based force generation (Grill et al., 2003). Thus, as opposed to pronuclear migration and centration/rotation, where length-dependent forces participate in driving centrosome movements, cortically driven forces drive metaphase/anaphase spindle positioning.
Interestingly, the spindle severing experiments also revealed that the posterior spindle pole moves towards the cortex with a higher velocity as compared to the anterior one and that this difference is under A-P polarity control (Figure 6; Grill et al., 2001). This indicated that net cortical pulling forces acting on the posterior spindle pole are higher than those on the anterior one, explaining the root of posterior spindle displacement. Further experiments demonstrated that a higher number of active force generating units functioning at the posterior cortex is responsible for such an imbalance in pulling forces (Grill et al., 2003). Interestingly, the sites of active force generation can be visualized as membrane invaginations upon partial disruption of the acto-myosin network (Redemann et al., 2010). Such invaginations are dependent on microtubules and occur more frequently at the posterior side in a polarity dependent manner.

CORTICAL FORCE GENERATOR COMPLEX

During the last decade or so, mutational analyses and RNAi-based screens lead to the identification of components involved in the generation of pulling force
during mitosis. These studies revealed the existence of a conserved force generator protein ensemble located at the cell cortex (Figure 7). The \( \alpha \)-subunits of two heterotrimeric G proteins have been identified as members of these complexes (Afshar et al., 2004; Colombo et al., 2003; Gotta and Ahringer, 2001; Grill et al., 2003). This is illustrated by the fact that simultaneous depletion of the two partially redundant Go subunits GOA-1 and GPA-16 impairs pulling forces and leads to equal first cell division (Colombo et al., 2003; Gotta and Ahringer, 2001). These Go proteins form a so-called ternary complex together with the two nearly identical GoLoco containing proteins GPR-1 and GPR-2 (hereafter referred to collectively as GPR-1/2) and the coiled-coil protein LIN-5 (Colombo et al., 2003; Couwenbergs et al., 2007; Gönczy et al., 2000; Gotta and Ahringer, 2001; Gotta et al., 2003; Nguyen-Ngoc et al., 2007; Park and Rose, 2008; Srinivasan et al., 2003; Tsou et al., 2003). Similarly to double Go depletion, inactivation of GPR-1/2 or LIN-5 function results in diminished pulling forces and equal cell division (Colombo et al., 2003; Couwenbergs et al., 2007; Gotta et al., 2003; Nguyen-Ngoc et al., 2007; Srinivasan et al., 2003). Biochemical studies established that the GDP-bound Go proteins associate with GPR-1/2, which in turn binds to LIN-5 (Couwenbergs et al., 2007; Nguyen-Ngoc et al., 2007; Park and Rose, 2008). Apart from providing the basis of force generation during mitosis, the ternary complex also contributes to centration/rotation (Goulding et al., 2007; Park and Rose, 2008).

The analysis of immunostained embryos revealed that some components of the ternary complex exhibit a non-uniform cortical localization during mitosis. While the cortical levels of GOA-1 and GPA-16 seem to be similar throughout the cell cortex (Afshar et al., 2004; Afshar et al., 2005; Gotta and Ahringer, 2001; Miller and Rand, 2000; Park and Rose, 2008), GPR-1/2 displays a slight asymmetry in anaphase, showing higher enrichment at the posterior (Colombo et al., 2003; Gotta et al., 2003; Tsou et al., 2003 and Figure 29). As far as LIN-5 is concerned, ~50% of the embryos exhibit higher relative posterior cortical enrichment, as compared to the anterior cortex, while the rest show high levels on both cortices of the cell (bipolar pattern) (Park and Rose, 2008). Why LIN-5 does not mirror the localization of GPR-1/2 is so far unresolved. During centration/rotation, both LIN-5 and GPR-1/2 are more enriched on the anterior cortex, correlating with the direction of force at that stage (Park and Rose 2008). Extra copies of GPR-1 in a transgenic lines expressing YFP-
GPR-1 result in exaggerated centrosomal movements during centration/rotation and to increased net pulling forces at anaphase (Redemann et al., 2011). This suggests that tight regulation of GPR-1/2 levels is crucial for the establishment of proper pulling forces.

How can the ternary complex generate cortically based force along astral microtubules? Dynein activity is crucial for this to occur. Embryos with diminished dynein activity or depleted of the associated proteins LIS-1 and DYRB-1 exhibit diminished pulling forces during mitosis (Couwenbergs et al., 2007; Nguyen-Ngoc et al., 2007). Importantly, the cortical localization of the dynein heavy chain DHC-1 and that of LIS-1 depend on the ternary complex (Nguyen-Ngoc et al., 2007). Furthermore, LIN-5 and GPR-1/2 co-immunoprecipitates with the dynein complex, with GPR-1/2 being dispensible for LIN-5-dynein association, whereas LIN-5 is needed for forming the GPR-1/2-dynein complex (Nguyen-Ngoc et al., 2007). The current model posits that mirystoylated Gα anchors GPR-1/2 associated with LIN-5 to the plasma membrane, thus creating a bridge between the dynein complex and the plasma membrane (Figure 7).

![Figure 7. Model of cortical force generation. See text for details. Adapted from Kotak and Gönczy, 2013.](image)

A convenient phenotypic readout of asymmetric force generation during anaphase is the vigorous oscillation of the posterior spindle pole that takes place as posterior spindle displacement is occurring (Figure 20A). Embryos lacking one copy of the Gα proteins fail to undergo vigorous posterior oscillation, whereas spindle
displacement is not affected and the cell division remains unequal as in the wild type (Afshar et al., 2005). Similarly, gradual decrease of the levels of the dynein light intermediate chain DLI-1 or of GPR-1/2 prevents posterior spindle pole oscillation, whilst the division is unequal (Pecreaux et al., 2006). This suggests that different thresholds in the levels of cortical force generator components operate for spindle displacement and for posterior oscillation, with the latter requiring more than the former.

How could dynein exert pulling force on astral microtubules? Two scenarios can be envisaged. First, dynein could function as a molecular motor by attempting to move towards the minus end of microtubules; however, dynein being anchored to the cortex, this attempt generates a pulling force on the astral microtubule instead. Alternatively, dynein could serve as coupling device that holds onto the microtubule plus ends and the pulling force generated by virtue of microtubule depolymerization. Modeling experiments of spindle displacement revealed that each force generator can exert a force of ~50 pN, which is in the range of that generated by a single depolymerizing microtubule, but almost a magnitude higher than the force deployed by a single dynein motor (Kozlowski et al., 2007). This suggests that dynein rather may rather serve as a coupling device and that the driving force for spindle displacement is generated by microtubule depolymerization. This is in accordance with the finding that preventing microtubule dynamics leads to the diminution of pulling forces (Nguyen-Ngoc et al., 2007). Supporting this model, purified dynein from yeast bound to the edge of micro-fabricated chambers captures microtubules and promotes microtubule catastrophe, thereby resulting in microtubule depolymerization (Laan et al., 2012).

REGULATION OF FORCE GENERATION

*Regulation exerted by individual proteins*

Several proteins have been identified that influence the force generation machinery (Figure 8). As expected, the asymmetric enrichment of GPR-1/2 is under the control of A-P polarity (Colombo et al., 2003; Gotta et al., 2003; Park and Rose, 2008; Tsou et al., 2003). Regulation at the level of the heterotrimeric G-proteins is also important. Single depletion of GPA-16 or of GOA-1 results in reduced GPR-1/2
cortical signal (Afshar et al., 2005), whereas double depletion of these proteins leads to the absence of GPR-1/2 at the cortex (Colombo et al., 2003) and to equal cell division. In the absence of RIC-8, a protein acting as a Guanine nucleotide exchange factor (GEF) for GOA-1 and that is needed for cortical GPA-16 enrichment, cortical GPR-1/2 recruitment is reduced (Afshar et al., 2004). A G-protein activator factor (GAP) has also been shown to modulate force generation at the level of GOA-1. Indeed, loss of the GAP protein RGS-7 leads to lower net pulling forces on the anterior side (Hess et al., 2004). In other systems, Gα proteins associate with β and γ subunits to form an inactive complex. In C. elegans, the β subunit GPB-1 and the γ subunit GPC-2 negatively regulate spindle positioning, as depletion of either of these two components results in excess centrosome movements during centration/rotation and in higher net pulling forces during anaphase (Afshar et al., 2004; Afshar et al., 2005; Tsou et al., 2003). This increase in pulling forces correlates with increased cortical GPR-1/2 levels at the anterior in gpb-1(RNAi) embryos during centration/rotation (Thyagarajan et al., 2011; Tsou et al., 2003). Importantly, depletion of the Gα components rescues the excess movements observed in gpb-1(RNAi) (Tsou et al., 2003), indicating that GPB-1 acts via negative regulation of the Gα proteins, and not through an independent mechanism.

**Figure 8. Regulators of force generation in C.elegans one-cell stage embryos.**

Regulation occurs at the level of the entire ternary complex (yellow), at the level of the Gα proteins (purple), influencing GPR-1/2 and LIN-5 (orange) or impinging on LIN-5 per se (blue). Positive regulators are shown in green, negative regulators in red. Disruption of
polarity can lead to increase or decrease in pulling forces, depending on which complex is compromised. See text for details.

Regulation occurs also at level of LIN-5 and GPR-1/2 distribution (Figure 8). The DEP-domain protein LET-99 is enriched in a posterior-lateral band and negatively regulates pulling forces during centration/rotation and anaphase (Krueger et al., 2010; Tsou et al., 2002; Tsou et al., 2003). In wild type embryos, GPR-1/2 and LIN-5 signals are lowest in the region where LET-99 is highest and, suggestively, let-99(RNAi) leads to higher than normal anterior GPR-1/2 and LIN-5 levels during centration/rotation and to uniform distribution at anaphase (Krueger et al., 2010; Park and Rose, 2008; Tsou et al., 2003). How the absence of two lateral bands of LET-99 at the posterior can have an impact on the anterior cortex is an intriguing and so far unresolved question.

Two kinases and one phosphatase are also part of the list of proteins that impinge on the force generation complex. Thus, PKC-3-mediated phosphorylation of four LIN-5 Ser residues has been shown to have an impact on force generation (Galli et al., 2011). In accordance with the anterior localization of PKC-3, immunostaining with phospho-specific antibody recognizing one of the four Ser residues revealed that P-LIN-5 decorates the anterior cortex of the cell. Moreover, LIN-5 phosphorylation by PKC-3 is dependent on the mitotic master regulator CDK-1. The phosphorylation status of these residues appear to be functionally relevant, as expression of non-phosphorylatable LIN-5 4A in the absence of endogenous LIN-5 leads to exaggerated oscillatory movement during centration/rotation and to an increase in anterior pulling forces. Conversely, introducing phosphomimetic mutations causes decreased oscillation during mitosis, although this experiment was performed at 16°C, where forces have been demonstrated to be lower (Johnston et al., 2008). Of note, this phenotype differs from lin-5(RNAi), as the division remains unequal, indicating that PKC-3-mediated phosphorylation is not the sole mechanism ensuring proper LIN-5 distribution and activity (Galli et al., 2011).

The casein kinase Iγ CSNK-1 functions as another regulator of LIN-5 and GPR-1/2 levels, as well as of GPR-1/2 asymmetry (Panbianco et al., 2008). csnk-1(RNAi) leads to excess centrosome movements during centration/rotation and to higher pulling forces at anaphase. GPR-1/2 and LIN-5 levels are increased in embryos depleted of CSNK-1. CSNK-1 is enriched in the anterior cell membrane, and it is
necessary for the posterior enrichment of its potential substrate, the PI(4)P5 kinase PPK-1. PPK-1 generates PtIns(4,5)P2 and it is plausible that PPK-1 influences spindle positioning by regulating the distribution ofPIP2 in an asymmetric manner. However, such asymmetry has not been reported using the PIP2 biomarker GFP-PH(PLC1d1) (Audhya et al., 2005). It is speculated that creating homogenous PPK-1 distribution in csnk-1(RNAi) changes the lipid composition in the anterior, thus influencing GPR-1/2 levels, but how such regulation may occur at the molecular level remains to be investigated. The identification of the potential molecular link between phospholipid composition and the distribution of the force generator complex will definitely be an exciting discovery.

The phosphatase PPH-6 and its associated partner SAPS-1 positively regulate force generation (Afshar et al., 2010). Depletion of pph-6 or saps-1 results in decreased GPR-1/2 and LIN-5 cortical levels, reduced posterior pulling forces, as well as in the lack of posterior spindle pole oscillations. How the action of this phosphatase is executed mechanistically is not yet known, but, interestingly, the PPH-6/SAPS-1 complex seems to act downstream of CSNK-1, as simultaneous depletion of saps-1 and csnk-1 results in phenotypes analogous to that of saps-1(RNAi) (Afshar et al., 2010).

Regulation by more general mechanisms

Regulation by intracellular trafficking

Intracellular trafficking enables exchange between different endomembrane compartments within a eukaryotic cell. Intracellular trafficking involves the secretory, the endocytic and the recycling pathways (reviewed in Doherty and McMahon, 2009; Glick and Nakano, 2009; Mayor and Pagano, 2007; Stolz and Wolf, 2010). The main components of the secretory pathway are the endoplasmic reticulum (ER) and the Golgi network, which are responsible for the delivery of proteins to the plasma membrane. The endocytic pathway is involved in the uptake of molecules from the extracellular space or from the plasma membrane. The recycling pathway is responsible for the replenishment of molecules onto the plasma membrane. There are several endocytic pathways, including the clathrin mediated endocytosis pathway. In this case, clusters of cargos molecules are surrounded with clathrin and its adaptor
proteins. Clathrin heavy chain, by its remarkable triskelion structure, is able to self-assemble into a structure that allows the invagination and finally the budding of these clathrin-coated pits from the plasma membrane (reviewed in Brodsky et al., 2001; Kirchhausen, 2000). The clathrin coated vesicles then fuse with the early endosomes where the decision is made concerning the next step; the vesicles can undergo lysosome-mediated degradation, be transported to the Golgi network or be recycled to the plasma membrane (reviewed in Bonifacino and Rojas, 2006; Grant and Donaldson, 2009; Hsu and Prekeris, 2010; Johannes and Popoff, 2008).

Several instances are known at present where intracellular trafficking is important for various processes in the C. elegans zygote. Polarity, for instance, is influenced by some of the component of the endosomal network. Thus, dynamin, a protein taking part in an early step of endocytic vesicle formation and in membrane fission, is needed for proper polarity maintenance (Nakayama et al., 2009). Upon dyn-1 depletion, embryos display decreased level of PAR-6 in the anterior, concomitant with an expansion of the PAR-2 domain. On the other hand, depletion of RAB-11, a protein that functions in recycling endosomes, cause the opposite effect, with an expansion of the PAR-6 domain and a smaller PAR-2 domain (Zhang et al., 2008). Early endosomes are also implicated in regulating polarity. Interfering with the early endosomal protein RAB-5 results in a subtle change in cortical dynamics of GFP-PAR-6, but without alteration in PAR-2 localization (Hyenne et al., 2012). Interestingly, both early and recycling endosomes, as well as the ER are enriched at the anterior side of the zygote (Andrews and Ahringer, 2007; Balklava et al., 2007; Poteryaev et al., 2005). The asymmetric enrichment of these components is A-P polarity dependent. Moreover, the anterior PAR complex and CDC-42 regulate endocytosis (Balklava et al., 2007). Together, these observations suggest that multidirectional regulation operates between the PAR proteins, the endosomal network and components influenced by intracellular trafficking.

What components are influenced by intracellular trafficking? An example is offered by the intracellular trafficking of the Gβ subunit GPB-1. As mentioned before, embryos depleted of GPB-1 show elevated GPR-1/2 levels on the anterior side of the cell (Tsou et al., 2003). Importantly, the levels of GPB-1 on the cell membrane is tightly regulated by intracellular trafficking ensuring low cortical GPB-1 levels during mitosis (Thyagarajan et al., 2011). GPB-1 associates both with early and
recycling endosomes, raising the possibility that GPB-1 cortical level is regulated by dynamic trafficking. In support of this hypothesis, inactivation of RAB-5 or of dynamin leads to polarity-dependent asymmetric GPB-1 accumulation in the cytoplasm, with more accumulation in the anterior side. It has been proposed that this asymmetric nature of GPB-1 trafficking influences GPR-1/2 cortical level, likely through modulating the availability of the active GDP-bound form of Gα proteins for interaction with GPR-1/2 (Thyagarajan et al., 2011).

Regulation by the acto-myosin network

The acto-myosin network influences several processes in the one-cell stage *C. elegans* embryo. As mentioned before, the acto-myosin network is responsible for setting up polarity in the zygote, thereby contributing to proper pulling forces and asymmetric cell division (Guo and Kemphues, 1996; Hill and Strome, 1988; Shelton et al., 1999). Experiments using time resolved drug treatments targeting the actin network were developed to address the involvement of the actin network in force generation after polarity establishment. Depolymerizing the actin network with Latrunculin A or Cytochalasin D leads to elevated pulling forces and increased spindle pole oscillations at the anterior (Afshar et al., 2010; Berends et al., 2013). In these conditions, however, the cortical levels of LIN-5 are not altered (Berends et al., 2013). It is thus clear that the actin network is a negative regulator of forces in the anterior during mitosis, but how this regulation occurs is not yet known. Interestingly, computer simulation experiments investigating the nature of spindle displacement predicted that lowering cortical rigidity, i.e. the strength of the acto-myosin network initially increases spindle pole oscillation, whereas further decrease in the rigidity dampens oscillation (Kozlowski et al., 2007). However, the involvement of the acto-myosin network in regulating centration/rotation might differ from its role during anaphase. Intriguingly, cortical GFP-NMY-2 foci display short-range anterior-directed movements during centration/rotation that were implicated in force generation (Goulding et al., 2007). In accordance with this hypothesis, partial depletion of *nmy-2* lowers the pace of centration/rotation. Furthermore, apart from the effect on mitotic pulling forces, NMY-2 organization at the time of female pronuclear migration is defective in embryos depleted of PPH-6 and SAPS-1 (Afshar et al.,
2010), possibly explaining the lack of pseudocleavage in these embryos. Whether this function of the PPH-6/SAPS-1 complex is linked to its role during spindle positioning or distinct from it remains to be investigated.

In summary, these regulatory mechanisms illustrate that the molecular events driving spindle positioning in *C. elegans* are thought to act strictly at the protein level and mRNA-based mechanisms have not been implicated in this process. This is surprising given that spatially restricted mRNA localization coupled with regulated translation have been shown to influence polarity-related processes in other systems (see next chapter). During the course of this thesis work, we provided evidences that the ‘protein-hegemony’ dogma does not entirely hold for one-cell stage *C. elegans* embryos, as localization of *lin-5* mRNA may be an important positive regulator of spindle positioning (see pages 55-84).

Asymmetric cell division and spindle positioning in other systems

Functional homologues of the ternary complex components are crucial for cortical dynein anchoring and thereby are involved in spindle positioning in other systems as well. Thus, the GPR-1/2 homologue Pins and the LIN-5 homologue Mud are implicated in asymmetric cell division of *Drosophila* neuroblasts (reviewed in Sousa-Nunes and Somers, 2013). Likewise, LGN (GPR-1/2 homologue) and NuMA (a LIN-5 homologue) function to position the mitotic spindle in human cells (reviewed in Kotak and Gönczy, 2013). A thorough understanding of the mechanisms governing spindle positioning will likely arise from comparing and contrasting findings from different systems. The mechanisms involved in asymmetric cell division and spindle positioning in *Drosophila* larval neuroblasts and in mammalian systems are introduced below (Figure 9).
Figure 9. Division of a Drosophila neuroblast and of a polarized mammalian cell

The components important for cleavage plane specification and cell fate determinant segregation in Drosophila neuroblast (A) and in a polarized mammalian cell (B). Details are in the text.

The Drosophila neuroblast

The diversity of the Drosophila central nervous system stems in part from several rounds of asymmetric cell divisions of neuroblasts in the developing brain. Type I neuroblasts undergo 12 cycles of asymmetric cell divisions before entering into quiescence in the embryo (Bossing et al., 1996; Hartenstein et al., 1987). Afterwards, in the larva, these cells divide again asymmetrically to give rise to neurons in the adult fly (Ito and Hotta, 1992). The embryonic neuroblasts delaminate from ectodermal epithelium, retaining their A-P polarity, while larval neuroblast gain apico-basal polarity in an unclear manner (reviewed in Campos-Ortega, 1993; Homem and Knoblich, 2012). A neuroblast divides asymmetrically and unequally: the larger apical cell, called neuroblast (NB) retains stem cell fate and gives rise to another round of asymmetric cell division, while the smaller basally located cell (named ganglion mother cell, GMC) differentiates into glial cell or neuron after subsequent divisions (Figure 9A).

The establishment of these fates is due to the asymmetric segregation of cell fate determinants along the apico-basal axis, driven by two apical complexes. The first complex comprises Par3 (Bazooka in Drosophila), Par6 and the atypical protein kinase C (aPKC) (Petronczki and Knoblich, 2001; Wodarz et al., 2000; Wodarz et al., 1999). This complex is linked to the second complex by the adaptor protein Inscutable (Insc) that interacts with both PAR-3 and Pins, a member of the second
apical complex (Kraut and Campos-Ortega, 1996; Kraut et al., 1996; Parmentier et al., 2000; Schaefer et al., 2001; Schober et al., 1999; Wodarz et al., 2000; Yu et al., 2000). Intriguingly, insc mRNA localizes as well to the apical complex, but this localization is dispensable for protein localization (Knoblich et al., 1999; Li et al., 1997). Thus the role of insc mRNA localization, if any, is unclear. The second apical complex comprises the heterotrimeric G-protein subunit $G\alpha_i$, Partner of Inscutable (Pins), Locomotive defects (Loco) and the coiled coil domain protein Mud (Bowman et al., 2006; Izumi et al., 2006; Siller et al., 2006). Pins and Loco function as GDI for $G\alpha_i$ and thus stabilize the GDP-bound form of $G\alpha_i$ (Parmentier et al., 2000; Schaefer et al., 2001; Schaefer et al., 2000; Yu et al., 2000; Yu et al., 2005), thereby promoting its activity. The Pins-$G\alpha_i$ complex is needed for proper spindle orientation (Schaefer et al., 2000; Yu et al., 2002). Furthermore, depleting the $G\beta$ protein, $G\beta_{13F}$ or the $G\gamma$ subunit $G\gamma_1$ also results in severe defects in asymmetric cell division (Fuse et al., 2003; Schaefer et al., 2001; Yu et al., 2003). This implicates that, similarly to *C. elegans*, $G\beta$ proteins influence spindle positioning in *Drosophila*, but whether the mechanisms are the same remains unclear. Interestingly, unlike in *C. elegans*, the $G\alpha_i$-Pins complex is necessary for proper polarity in neuroblasts (Parmentier et al., 2000; Schaefer et al., 2000; Yu et al., 2000; Yu et al., 2005).

What are the downstream effectors whose asymmetric distribution is regulated by the PAR-$G\alpha_i$ complex? These include the proteins Prospero (Pros), Brain tumor (Brat), Staufen, Numb and Partner of Numb (Pon), which all segregate in the GMC during division and play crucial roles in defining the GMC fate. The homeobox transcription factor Pros serves as a binary switch in the GMC, where it represses genes responsible for proliferation and self-renewal (such as cyclin E, cdc25/String) and switches on genes required for differentiation (Choksi et al., 2006; Doe et al., 1991; Hirata et al., 1995; Knoblich et al., 1995; Li and Vaessen, 2000; Southall and Brand, 2009; Spana and Doe, 1995). Interestingly, apart from Pros protein, *pros* mRNA is also enriched in the GMC after cell division, driven by the asymmetric segregation of the mRNA-binding protein Staufen that binds to the 3’ UTR of *pros* mRNA (Broadus et al., 1998; Schuldt et al., 1998). Brat likewise shows enrichment in the GMC where it downregulates Myc expression (Bello et al., 2006; Betschinger et al., 2006; Lee et al., 2006b). The phosphotyrosine binding protein Numb together
with its adaptor protein Pon inhibit Notch signaling in the GMC, thereby contributing to its fate (Lu et al., 1998; Rhyu et al., 1994; Uemura et al., 1989).

What are the mechanisms ensuring the asymmetric segregation of these components? Two pathways have been identified. First, the basal distribution of Pros, Brat and Staufen is regulated by their interaction with the basally enriched adaptor protein Mira (Figure 1D, (Betschinger et al., 2006; Ikeshima-Kataoka et al., 1997; Lee et al., 2006b; Matsuzaki et al., 1998; Schuldt et al., 1998; Shen et al., 1997). The N-terminal of Mira interacts with Insc, while Pros, Brat and Staufen bindings sites were mapped in the Mira C-terminal region (Fuerstenberg et al., 1998; Shen et al., 1998). Interestingly, mira mRNA is also asymmetrically localized in neuroblast, but, surprisingly, it is enriched in the apical side of the cell (Schuldt et al., 1998). Given the opposite distribution pattern of Mira mRNA and protein, it is postulated that Mira translation occurs on the apical side and that newly synthesized Mira protein rapidly relocates to the basal cortex (Erben et al., 2008). Mira is enriched in the basal cortex from prometaphase onwards in a manner that depends on actin, myosin II (Zipper) and myosin VI (Jaguar) (Barros et al., 2003; Petritsch et al., 2003; Shen et al., 1997), thereby promoting the asymmetric distribution of its cargos, Pros, Brat and Staufen. After mitosis, the release of the cargo proteins is achieved through the degradation of Mira (Matsuzaki et al., 1998; Schuldt et al., 1998; Shen et al., 1998). The second pathway ensures correct distribution of Numb by asymmetrically segregating the adaptor protein Pon (Lu et al., 1998). In the beginning of mitosis, Pon is distributed throughout the whole cell cortex, and becomes gradually enriched in the basal cortex (Lu et al., 1999). The apical clearance of Pon is mediated by phosphorylation exerted by Polo kinase (Wang et al., 2007). Although insc mutations result in aberrant Mira and Pon distribution at metaphase, this defect is corrected at telophase by a mechanism known as telophase rescue (Peng et al., 2000; Schober et al., 1999; Wodarz et al., 1999).

How is polarity linked to the segregation of Mira and its downstream effectors? Neuroblasts harboring mutation in the gene lethal giant larvae (Lgl) exhibit normal polarity but disturbed segregation of Mira and Pon (Ohshiro et al., 2000; Peng et al., 2000), suggesting that Lgl is an important component. aPKC-mediated phosphorylation plays a crucial in regulating Lgl localization. Prior to mitosis, Lgl is associated with Par6 and aPKC in the apical cortex. Upon aPKC activation by the
mitotic Ser/Thr kinase Aurora A, aPKC phosphorylates Lgl, which results in its removal from the apical cortex (Betschinger et al., 2005; Wirtz-Peitz et al., 2008). In the absence of Lgl, Par3 associates with the complex that recruits and phosphorylates Numb. Phospho-Numb relocates to the cytoplasm and then with the help of Zipper finally becomes enriched in the basal cortex (Lee et al., 2006a; Smith et al., 2007; Strand et al., 1994; Wang et al., 2006; Wirtz-Peitz et al., 2008).

To ensure the proper segregation of the above-mentioned cell fate determinants, the mitotic spindle must be aligned along the apico-basal axis. Following delamination from the epithelium, the spindle in the neuroblast becomes oriented along the A-P axis by undergoing a 90° rotation (Kaltschmidt et al., 2000). As the position of the centrosomes remains aligned along the apico-basal axis following the first cell division, subsequent NB divisions do not require spindle rotation (Rebollo et al., 2009). Reduced levels of Gαi, Pins, Mud or of the lissencephaly related protein Lis1 cause spindle orientation defects, while polarity is unaffected in these cases, indicating that these components act more specifically to regulate spindle positioning (Bowman et al., 2006; Cabernard and Doe, 2009; Izumi et al., 2006; Parmentier et al., 2000; Schaefer et al., 2001; Schaefer et al., 2000; Siller et al., 2006; Siller and Doe, 2008; Yu et al., 2000). The centrosomal protein Ana2 contributes to Pins-independent Mud localization through an interaction with the dynein light chain protein Cut up (Wang et al., 2011). Moreover, the Pins-interacting PDZ domain protein Canoe is also important for cortical Mud recruitment by interacting with RanGTPase through its Ras association domain (Speicher et al., 2008; Wee et al., 2011). A model operating with cortically anchored dynein/dynactin exerting a pulling force on astral microtubules emanating from the apical centrosome has been put forth, reminiscent of the spindle positioning mechanisms acting in the C. elegans zygote (reviewed in Siller and Doe, 2009).

The missegregation of the cell fate determinants described above lead to deleterious effects, such as overproliferation and tumor formation (Betschinger et al., 2006; Lee et al., 2006b). Based on this and related findings, it is believed that correct cell fate determination executed by asymmetric cell division functions as a tumor suppressor mechanism.
Mammalian systems

Although less well documented as compared to invertebrate model organisms, asymmetric cell division and spindle positioning have been investigated during mammalian development and in cell culture (Figure 9B).

Asymmetric cell divisions occur extensively in the developing vertebrate cerebral cortex. The progenitor cells in the ventricular zone of the brain can divide symmetrically, giving rise to two progenitors, or asymmetrically leading to the birth of one progenitor cell and of one cell that differentiates into a neuron. The symmetric divisions are present mostly during early embryogenesis, while the asymmetric cell divisions occur thereafter. Spindle orientation with respect to the plane of the neuroepithelium does not always correlate with the division being symmetric or asymmetric (reviewed in Huttner and Brand, 1997). In some instances, divisions perpendicular to the neuroepithelium lead to asymmetric cell division (Chenn and McConnell, 1995; Haydar et al., 2003; Noctor et al., 2004), but divisions parallel to the neuroepithelium have also been observed in both cases (Konno et al., 2008). The players involved in spindle positioning in invertebrate systems seem to be conserved in mammals. Thus, the progenitors are polarized through a Par3/Par6/aPKC-rich apical domain (Manabe et al., 2002). Par3 interacts with Numb and drives its apical enrichment (Bultje et al., 2009). In the absence of Numb or of its interacting protein Numblike, overproduction of neurons in the forebrain or reduction in the number of differentiated motoneurons is observed (Petersen et al., 2004; Zhong et al., 1996; Zilian et al., 2001). Insutable is important for establishing the plane of division in the retina, where the spindle is normally oriented perpendicular to the neuroepithelium, as interfering with Insc causes the plane of division to be parallel to the neuroepithelium and disrupts cell fate (Zigman et al., 2005). Interfering with the βγ subunits of the heterotrimeric G proteins alters the division plane and leads to overproduction of differentiated neurons (Sanada and Tsai, 2005). Here, AGS3, a homologue of GPR-1/2 and Pins promotes dissociation of Gβγ from Gα, and reduction of AGS3 levels phenocopies the depletion of Gβγ (Sanada and Tsai, 2005). Similarly, depletion of LGN, a protein related to GPR-1/2, and Pins, also leads to spindle misorientation (Konno et al., 2008; Shitamukai et al., 2011). Moreover, centrosomal proteins also influence spindle positioning. Thus, loss of the human ortholog of the abnormal spindle gene Aspm or of the Lis-1-interacting protein mNde1 lead to defects in
cleavage plane orientation, to a progressive decrease in the number of progenitor cells and finally to a smaller cerebral cortex, resulting in a condition termed microcephaly (Bond et al., 2002; Feng and Walsh, 2004).

An attractive system to study asymmetric spindle positioning in mammals is the developing mouse skin (Figure 1E; reviewed in Fuchs and Raghavan, 2002). During early development, cell divisions parallel to the underlying membrane produce a large number of progenitor cells that divide later in a perpendicular manner to produce basal and suprabasal cells. The former retains proliferative potential, while the latter differentiates into a specific cell type. Perpendicular divisions thus drive morphogenesis (stratification) of the skin. Mouse inscutable (mInsc) interacts at the apical membrane with Par3 and LGN, and they all form an apical crescent in mitotic cells (Lechler and Fuchs, 2005). The choice of a given cell to undergo symmetric or asymmetric cell division is determined by the apical localization of mInsc and NuMA (Poulson and Lechler, 2010). Furthermore, depleting LGN, NuMA or dynactin leads to aberrant spindle orientation and to defects in cell fate determination via the loss of the stratification-promoting Notch signaling (Williams et al., 2011).

Cell biological studies allied with the power of biochemical techniques in cultured cells largely contributed to understanding the mechanisms ensuring spindle positioning. As mentioned before, the ternary complex related to the Gα-GPR-1/2-LIN-5 and the Gαi/Pins/Mud complex is composed of Gαi-LGN-NuMA in human cells. This complex is important in regulating spindle positioning in polarized vertebrate cells (Du and Macara, 2004; Peyre et al., 2011; Williams et al., 2011; Zheng et al., 2010) and in non-polarized HeLa cells relative to a fibronectin substratum (Kiyomitsu and Cheeseman, 2012; Kotak et al., 2012; Woodard et al., 2010). The cortical enrichment of NuMA is prevented by phosphorylation by the mitotic kinase CDK1. The proportion of phosphorylated NuMA decreases as CDK1 activity drops at the metaphase to anaphase transition, leading to elevated non-phosphorylated NuMA in anaphase. This provides a temporal switch for establishing proper cortical dynein levels and robust spindle positioning (Kotak et al., 2013). Moreover, the polo-like kinase Plk1 negatively regulates dynein by inhibiting its interaction with the ternary complex during metaphase (Kiyomitsu and Cheeseman, 2012). Like in C. elegans, the downstream effector dynein is linked to the ternary complex through interaction with NuMA (Kiyomitsu and Cheeseman, 2012; Kotak et
al., 2012; Merdes et al., 1996), and it is believed that the sole function of the ternary complex is to anchor dynein to the cortex (Kotak et al., 2012).
1B. Polarized mRNA localization and translational control

Zygotic transcription is usually not active during the early steps of embryogenesis, and localization of maternal mRNAs and the regulation of their translation are widespread mechanisms utilized to drive early embryonic processes in many systems. The importance of mRNA localization can be appreciated for instance by the fact that ~70% of the 3370 mRNAs analyzed in a large scale in situ hybridization experiment in Drosophila embryos exhibit non-uniform intracellular localization (Lecuyer et al., 2007).

Below, the mechanisms driving such mRNA-related processes will be discussed, focusing on the Drosophila and the Xenopus oocytes, as well as on ash1 mRNA localization in budding yeast. Finally, regulation of mRNA translation and a few instances of asymmetrically localized mRNAs in early C. elegans embryos will be introduced.

The Drosophila oocyte

Probably the best studied system where the role of mRNA localization and translational control has been investigated is the Drosophila oocyte. The Drosophila oocyte develops within an egg chamber, where an oocyte is surrounded by 15 nurse cells (Figure 10, reviewed in King, 1970). The transcriptionally active nurse cells endow the oocyte with proteins and mRNAs through intercellular cytoplasmic bridges called ring canals. At the end of oogenesis the nurse cells undergo apoptosis. The localization of four mRNAs, oskar (osk), bicoid (bcd), nanos (nos) and gurken (grk) has been extensively studied, as these mRNAs are localized to different part of the oocyte and are responsible for establishing the main axes of the future embryo (reviewed in Bastock and St Johnston, 2008; Becalska and Gavis, 2009; Lasko, 2012). The localization of bcd and grk mRNA is essential to specify the antero-posterior and dorso ventral axes, respectively (Berleth et al., 1988; Driever and Nusslein-Volhard, 1988; Frohnhöfer et al., 1986; Neuman-Silberberg and Schupbach, 1993). osk is a germline determinant (Ephrussi et al., 1991; Ephrussi and Lehmann, 1992; Lehmann and Nusslein-Volhard, 1986), whereas nos is involved in germline an abdominal development (Gavis and Lehmann, 1992; Lehmann and Nusslein-Volhard, 1991;
Wang et al., 1994; Wang and Lehmann, 1991). A common theme for an initial step in loading these mRNAs is the dynein-dependent transport of grk, bcd and osk mRNAs from the nurse cells to the oocyte (Cha et al., 2001; Clark et al., 2007; Mische et al., 2007; Schnorrer et al., 2000).

**Figure 10. mRNA localization in the Drosophila oocyte**

Nuclei (blue) and microtubules (green) are depicted. mRNA distribution of oskar, nanos, bicoid and gurken are shown.

Once in the oocyte, bcd mRNA becomes enriched in the anterior part through several steps, including initial loading and anchoring (St Johnston et al., 1989). The initial anterior enrichment is driven by the RNA-binding protein Exuperantia (Exu), whose activity is under the control of the kinase Par1 (Berleth et al., 1988; Cha et al., 2001; Mische et al., 2007; Riechmann and Ephrussi, 2004). Staufen is likewise necessary for anterior enrichment of bcd mRNA by binding to its 3’ UTR (Ferrandon et al., 1994; St Johnston et al., 1989) and Swallow is important in a subsequent anchoring step of the pathway by regulating the actin cytoskeleton (Weil et al., 2010). The endosomal sorting complex ESCRT-II is necessary for proper bcd mRNA enrichment, with the ESCRT-II complex subunit VPS36 binding directly to the bcd 3’ UTR (Irion and St Johnston, 2007). This regulation, however, might not be linked to endosomal sorting, as the ESCRT-I and III complexes that likewise function in endosomal sorting do not regulate bcd mRNA localization (Irion and St Johnston, 2007). bcd mRNA is under translational repression until fertilization, and the Bicoid protein gradient is established thereafter solely as a result from the mRNA localization and local translation. In the embryo, Pumilio negatively controls bcd translation by binding to its 3’ UTR and a similar mechanisms might take place in the oocyte to ensure temporal regulation of bcd translation (Gamberi et al., 2002).
turn, Bcd protein is important to regulate the P-A protein gradient of the transcription factor Caudal (Cad) by repressing the translation of cad mRNA (Dubnau and Struhl, 1996; Rivera-Pomar et al., 1996). Bcd binds to the cad 3’ UTR and recruits the 4E homology protein 4EHP, leading to the formation a translationally-inactive circularized cad mRNA (Cho et al., 2005; Hernandez et al., 2005).

osk mRNA accumulates at the posterior pole of the oocyte, where Osk protein translation starts at mid-oogenesis. Oskar nucleates the formation of a specialized region of the cytoplasm, termed the pole plasm, where large ribonucleoprotein complexes form and include posterior and germ cell determinants, such as Nos. In the case of osk mRNA, localized mRNA is coupled with spatially restricted translational regulation. Localization requires the 3’ UTR and also signals stemming from the activity of the exon-junction complex that drive splicing (Hachet and Ephrussi, 2001, 2004; Kim-Ha et al., 1993; Newmark and Boswell, 1994). osk mRNA lacking introns can localize solely in the presence of the endogenous osk transcript via RNA-RNA interactions through the 3’ UTRs (Jambor et al., 2011). After its initial dynein-mediated enrichment has been established, the plus end motor kinesin, the actin cytoskeleton, the endosomal network and one of the Oskar isoforms itself together contribute to the posterior anchoring of osk mRNA (Rongo et al., 1995; Tanaka and Nakamura, 2011; Vanzo et al., 2007; Zimyanin et al., 2008). The RNA-binding protein Staufen is needed for osk mRNA localization and for translational activation by interacting with the osk 3’ UTR (Braat et al., 2004; Kim-Ha et al., 1995; Micklem et al., 2000). Translational repression during early oogenesis requires genes implicated in the RNAi pathway (armitage, zucchini, quash) and the polypirimidine tract binding protein PTB that binds to the osk 3’ UTR (Besse et al., 2009; Cook et al., 2004; Pane et al., 2007; Tomari et al., 2004). Later on, translational repression is executed by Bruno, a protein that binds to the osk 3’ UTR (Kim-Ha et al., 1995; Snee et al., 2008). Bruno recruits the eIF4E binding protein Cup, leading to the accumulation of the CCR4 deadenylase complex, resulting in the shortening of the osk poly-A tail (Igreja and Izaurralde, 2011; Nakamura et al., 2004). It has been postulated that through this mechanism Cup protects osk mRNA from further degradation.

The posterior enrichment of nos mRNA is driven by the proteins Rumpelstilskin and Aubergine, which are associated with the nos 3’ UTR (Becalska
et al., 2011; Jain and Gavis, 2008). Since the posterior enrichment of nanos mRNA is not efficient (only 4% of all nos mRNA is localized (Bergsten and Gavis, 1999), translational regulation takes place to prevent Nos protein from localizing to the anterior. These regulatory mechanisms involve a 90 nt long element in the nos 3’ UTR termed the translational control element, or TCE (Cruc et al., 2000; Forrest et al., 2004). The secondary structure of the TCE is recognized by several translational repressors, including Glorund and Smaug (Kalifa et al., 2006; Nelson et al., 2004). Smaug interacts both with Cup and with Pop2, a subunit of the CCR4 deadenylase complex, thereby leading to translational repression of nos (Semotok et al., 2005; Zaessinger et al., 2006). Oskar counteracts the Smaug-mediated repression in the germ pole, leading to enrichment of Nos protein in the very posterior (Zaessinger et al., 2006).

*gurken* mRNA is associated with the oocyte nucleus that is initially located at the posterior side and relocates thereafter to the antero-dorsal part of the oocyte. *grk* mRNA localization is dynein-dependent and involves squid/hnRNP (Delanoue et al., 2007; Jaramillo et al., 2008). The localization signal lies in the coding region, which is necessary but not sufficient for anterodorsal enrichment (Lan et al., 2010; Van De Bor et al., 2005). *grk* mRNA is also under translation control. The eIF5B interacting protein Vasa is a translational activator, as oocytes mutant for vasa or expressing Vasa protein incapable of interacting with eIF5B, result in decreased Gurken protein level (Johnstone and Lasko, 2004; Styhler et al., 1998; Tomancak et al., 1998).

The *Xenopus* oocyte

The *Xenopus* oocyte is a polarized cell, possessing an animal and a vegetal pole (Figure 11). Oocyte maturation goes through six stages, referred to as Stage I-VI (reviewed in Dumont, 1972). Whereas mechanisms driving mRNA localization to the animal pole are not known, the enrichment of mRNAs in the vegetal pole is executed by two pathways: the early message transport organizer (METRO) pathway and the late pathway (Forristall et al., 1995; Kloc and Etkin, 1995).
Figure 11. mRNA localization pathways in the *Xenopus* oocyte

The early pathway acts through stages I-III (red), while the late pathway starts driving mRNAs distribution between stage III and VI (blue). Adapted from Mowry and Cote, 1999.

The METRO pathway localizes several mRNAs encoding RNA-binding proteins important for germline specification, such as the *nanos*-related *Xcat2* and *Xdaz1* (Forristall et al., 1995; Houston et al., 1998; Kloc and Etkin, 1995; Mosquera et al., 1993). Interestingly, the translation of these mRNAs can be repressed for several months (MacArthur et al., 1999). These mRNAs are associated with the mitochondrial cloud, or Balbiani body, a structure formed around the centrioles that is enriched with ER, Golgi and mitochondrial membranes (Heasman et al., 1984; Kloc et al., 1996). Dynein-mediated transport has been implicated in building up the Balbiani body (Heasman et al., 1984; Kloc et al., 2004; Kloc et al., 2002; Pepling et al., 1999), while the mRNAs are thought to be delivered there by diffusion and by entrapment mechanisms that can occur in the absence of microtubules and actin (Kloc et al., 1996). Some *cis*-elements have also been identified as being important for mRNA distribution, such as a 164-nt long region in the *Xcat2* 3’UTR that is necessary and sufficient for localization to the Balbiani body (Kloc et al., 2000).

The T-box transcription factor VegT and the TGF-β growth factor Vg1 are the best-studied examples of mRNAs localized by the late pathway (Stennard et al., 1996; Weeks and Melton, 1987; Zhang and King, 1996). VegT and Vg1 take part in mesoderm and endoderm formation (Dale et al., 1993; Horb and Thomsen, 1997; Thomsen and Melton, 1993). Unlike the METRO pathway, the late pathway utilizes microtubules to localize mRNAs (Yisraeli et al., 1990) and the final enrichment at the vegetal cortex is exerted through several steps. The initially homogenously distributed Vg1 mRNA becomes enriched on the smooth ER in the vegetal pole in a microtubule dependent manner (Deshler et al., 1997; Kloc and Etkin, 1998). Afterwards, Vg1 mRNA is transported to the cortex by the motor proteins kinesin I and II (Betley et al., 2004; Messitt et al., 2008; Yoon and Mowry, 2004). The connection between the
motor complex and the mRNA is thought to be mediated by Staufen that binds both Vg1 3’ UTR and kinesin I (Yoon and Mowry, 2004). When arrived to its final destination, actin-based anchoring through the protein Prrp ensures Vg1 mRNA localization at the vegetal cortex (Zhao et al., 2001).

ash1 mRNA localization in budding yeast

Among the ~30 mRNAs (Aronov et al., 2007; Shepard et al., 2003) that are actively transported to the bud, the mechanisms driving ash1 mRNA localization and translation are the best studied (Figure 12). The protein ASH1 (Asymmetric synthesis of HO) is enriched in the daughter cell after division and functions as a repressor of the HO endonuclease, thereby inhibiting mating type switching in this cell (Chartrand et al., 1999; Takizawa et al., 1997). Such asymmetric enrichment of ASH1 in the daughter cell is the end result of active mRNA transport to the bud during late anaphase coupled with translational repression (Figure 1A). Four cis-elements have been identified that are all sufficient for localization (Chartrand et al., 1999; Gonzalez et al., 1999): three of them are in the coding region, while the fourth overlaps with the coding and the 3’ UTR. All four elements fold in a stem-loop that is recognized by the RNA-binding protein She2p (Bohl et al., 2000; Long et al., 2000). She2p, via its binding partner She3p, is associated with the type V myosin Myo4p, forming the so-called locasome (Bohl et al., 2000; Long et al., 2000; Takizawa et al., 1997). As its name indicates, this complex is responsible for transporting cargos (in this case mRNAs) along the actin cytoskeleton (Takizawa et al., 1997; Zhao et al., 2001). Interestingly, the cortical transport of the endoplasmic reticulum is likewise mediated by She3p-Myo4p complex, and it may be linked with bud-directed mRNA trafficking (Estrada et al., 2003; Schmid et al., 2006). Furthermore, ASH1 protein is also crucial for ash1 mRNA localization, but whether this is the result of a direct or indirect interaction is not known (Gonzalez et al., 1999).
Translational repression of ash1 mRNA is exerted by two proteins, Puf6p and Khd1p. Puf6p belongs to the Pumilio family of RNA-binding proteins and interacts with the fourth localization element in the ash1 mRNA (Gu et al., 2004). *puf6* deletion results in reduced asymmetry of both the mRNA and the protein, indicating that translational repression is somehow linked to mRNA localization. The regulation by Khd1p acts via the first localization element and involves competition with eIF4G1 (Paquin et al., 2007). Khd1p overexpression results in mislocalized *ash1* mRNA and decreased Ash1p levels (Irie et al., 2002). An elegant regulatory circuit mediated by the casein casein kinase CK2 and the Yck1p kinase anchored at the bud tip releases ash1 mRNA from translational repression in a localized manner by phosphorylating Khd1p (Deng et al., 2008; Paquin et al., 2007).
Translational control during early *C. elegans* development

Although not as widespread as in other systems such as the *Drosophila* oocyte, translational control has been shown in some cases to be important for early developmental processes in *C. elegans* (Figure 13, reviewed in Hwang and Rose, 2010).

**Figure 13. Instances where translational regulation has been shown to be important in early *C. elegans* embryos**

Positive regulators (translational activators) of the mRNAs encoding the indicated proteins are shown in green, negative regulators (translational repressors) are in red. Note that in all cases, the mRNA is distributed uniformly in the embryo. See text for more details. A MEI-1 protein level regulated by SPN-2 and OMA-1. B MEX-5/6 regulation by POS-1. C Regulation of ZIF-1 by MEX-5/6, SPN-4 and POS-1. D APX-1 regulation by PIE-1. E GLP-1 regulation by SPN-4, MEX-5/6 and POS-1. F Regulation of PAL-1 exerted by MEX-3 and MEX-5/6. G NOS-2 regulation by MEX-3 and SPN-4 (M-3, S-4).

Interfering with the function of genes encoding the Nanos homologue *nos-3*, the Pumilio-related genes *fbf-1/2* and the Brat homologues *ncl-1, nhl-1, nhl-2* and *nhl-3* (collectively referred to as ‘Cebrats’) each suppress lethality of the *par-2(it5)* temperature sensitive mutants (Hyenne et al., 2008; Labbe et al., 2006). In *Drosophila*, homologues of these proteins act as translational repressors in the oocyte. It has been indicated that the regulation observed in *C. elegans* occurs through altering the levels of PAR-6, as *nos-3* mutants exhibit lower PAR-6 levels in the embryos (Pacquelet et al., 2008). How could a potential translational repressor lead to
decreased level of a given protein? NOS-3 represses translation of the E3-ligase subunit FEM-3, by binding to the 3’ UTR of fem-3 mRNA in the gonad (Kraemer et al., 1999). The FEM-3 based E3 ligase CBC-FEM-3 binds PAR-6 in vitro (Pacquelet et al., 2008). Thus, in the absence of NOS-3, increased levels of FEM-1 can direct more PAR-6 for proteasomal-mediated degradation presumably in the oocyte, thereby balancing out the effect caused by reduced PAR-2 function.

One example of translational regulation happening probably also in the oocyte that bears consequences in the early embryo is offered by the action exerted by SPN-2 on MEI-1 protein levels (Figure 13A). SPN-2 is an eIF4E binding protein whose depletion leads to defects in spindle orientation (Li et al., 2009). In spn-2 mutant embryos, the mitotic spindle in the zygote fails to align properly along the A-P axis, leading to problems in cleavage plane orientation. Interestingly, ectopic expression of MEI-1, a subunit of the katanin complex responsible for severing microtubules, was observed in spn-2 mutants. MEI-1 is needed for proper meiotic spindle assembly, but is normally eliminated from the zygote before mitotic spindle formation, thus ensuring proper spindle positioning (Clark-Maguire and Mains, 1994a, b; Srayko et al., 2000). Suggestively, reducing mei-1 levels suppressed the spindle orientation defects in spn-2 embryos, indicating that ectopic MEI-1 expression is causative of the phenotype. eIF4E binding proteins inhibit the assembly of the translational initiation complex, such that the regulation of MEI-1 levels is thought to act through translational repression. In support of this hypothesis, SPN-2, in complex with an RNA-binding protein, OMA-1 binds to the mei-1 3’ UTR (Li et al., 2009).

A bidirectional regulation takes place between MEX-5/6 and POS-1 (Figure 13B). As mentioned before, upon depletion of MEX-5/6, POS-1 is no longer restricted to the posterior lineage (Schubert et al., 2000). Mechanistically, this is thought to happen both at the level of pos-1 mRNA and POS-1 protein. As opposed to the wild type situation where pos-1 mRNA is enriched in P, as compared to AB, this is no longer observed in single mex-5 mutant embryos, while POS-1 protein is still enriched in the posterior cell (Tenlen et al., 2006). However, mex-5 and mex-6 double mutant embryos exhibit homogenous POS-1 protein distribution (Schubert et al., 2000). These observations argue that POS-1 protein level is also regulated by MEX-5/6 either at the level of translation or at the level of protein degradation. Hinting towards the latter possibility, POS-1 undergoes E3-ligase mediated degradation in the
somatic lineages by the CUL-2-ZIF-1 complex in a MEX-5/6 dependent manner (DeRenzo et al., 2003; Reese et al., 2000). One possibility that has been suggested is that MEX-5/6 functions as a translational activator of zif-1 mRNA, although this idea needs to be tested experimentally (Figure 13C). Regulation also exists from POS-1 to the MEX-5/6 complex. Thus, reduction of pos-1 function rescues phenotypic defects normally present in mex-5 mutant embryos (Tenlen et al., 2006). This argues that POS-1 is a negative regulator of MEX-5 (and potentially MEX-6), maybe through translational regulation, as it has been shown for POS-1 in the case of glp-1 mRNA (Ogura et al., 2003). Supporting this model, POS-1 interacts with the mex-6 3’ UTR (Tenlen et al., 2006).

At the end of the second cell division, ABa and ABp are born with identical developmental potential (Figure 3C) and signaling arising from P2 is responsible for the specification of the ABp fate (Bowerman et al., 1992). It turned out that the Notch signaling from P2 to ABp plays a key role in this process (reviewed in Evans and Hunter, 2005; Priess, 2005). How does this work at the molecular level? The spatial regulation of both the ligand and the receptor is the answer (Figure 13D and E). GLP-1, the Notch receptor, is solely present on the plasma membranes of ABa and ABp, while it is only at the P2 - ABp boundary that the Notch receptor APX-1 is present (Evans et al., 1994; Mickey et al., 1996). Both glp-1 and apx-1 mRNAs are homogenously distributed in the 4 blastomeres; therefore translational regulation must take place to achieve the observed non-uniform protein distribution. APX-1 protein is not detectable in pie-1 mutant embryos, indicating a role for PIE-1 in apx-1 mRNA translational activation (Mickey et al., 1996). Extensive regulation of the glp-1 mRNA has also been found, leading to its observed protein distribution. POS-1 and another RNA binding protein GLD-1, bind to glp-1 3’UTR elements, leading to its translational repression (Marin and Evans, 2003; Ogura et al., 2003). Thus, loss of pos-1 leads to GLP-1 enrichment in the posterior cells (Ogura et al., 2003). Furthermore, SPN-4 activates the translation of glp-1 mRNA by binding to an other region of the 3’ UTR of glp-1 mRNA, with the loss of spn-4 activity resulting in the absence of GLP-1 protein (Ogura et al., 2003). Furthermore, GLP-1 protein levels are diminished in mex-5/6 double mutant embryos (Schubert et al., 2000). Given the abovementioned degradation of POS-1 in the somatic blastomeres, SPN-4-MEX-5/6
mediated translational activation wins in the anterior cells, giving rise to the presence of GLP-1 protein only in those cells.

The homeobox protein PAL-1 is the C. elegans Caudal homologue. PAL-1 is required for the specification of the C and D fates and thus for proper hypodermal development (Waring and Kenyon, 1990, 1991). pal-1 mRNA is present only in the posterior nuclei at the 4-cell stage, while its mRNA is evenly distributed (Figure 13F; (Edgar et al., 2001; Lei et al., 2009), raising the possibility of another instance of translational regulation. PAL-1 protein is misdistributed in embryos lacking MEX-5/6 or MEX-3 (Huang et al., 2002; Schubert et al., 2000). Does PAL-1 distribution involve the 3’ UTR? Indeed, as a LacZ reporter construct harboring the pal-1 3’UTR recapitulates the posterior expression pattern in a mex-3-dependent manner (Hunter and Kenyon, 1996). Moreover, MEX-3 interacts with the pal-1 3’ UTR (Pagano et al., 2009). A model emerges where MEX-3, together with MEX-5/6, acts as translational repressor of pal-1 mRNA in the anterior blastomeres. Whether MEX-5/6 interact directly with pal-1 mRNA or act through MEX-3 remains an open question.

MEX-3 likewise regulates the restricted translation of NOS-2, a transcription factor important for the acquisition of P-cell fate (Figure 13G; Jadhav et al., 2008; Pagano et al., 2009; Subramaniam and Seydoux, 1999). MEX-3 binds to the nos-2 3’ UTR and represses its translation until the 28-cell stage, where NOS-2 appears in P4. Similarly to the case of pal-1, a repressor (MEX-3) and an activator (PIE-1) function in concert here to establish spatially restricted protein distribution. Apart from these proteins, SPN-4 also seems to play a role in regulating NOS-2 distribution, as ectopic NOS-2 is observed in spn-4 mutant embryos (Jadhav et al., 2008).

Polarized mRNAs in early C. elegans embryos

Polarized distribution of mRNAs is not frequent in C. elegans early embryos and the majority of mRNAs exhibits homogenous localization and thus falls into the group of so-called class I mRNAs (Figure 14A; Seydoux and Fire, 1994). However, several mRNAs have been identified as being polarized in embryos from the 2 to 28-cell stage. One group of such maternally provided mRNAs are the class II mRNAs that go from being homogeneously distributed in the zygote to being enriched in the P-lineage later during development (Figure 14B; Seydoux and Fire, 1994). This process
potentially involves degradation mechanisms acting in the somatic cells. Examples of such mRNAs are *cey-2*, *skn-1* and the already mentioned *pos-1* (Seydoux and Fire, 1994; Tabara et al., 1999). Interestingly, *glp-1* mRNA also shows enrichment in the P-lineage in 8-cell stage embryos, suggesting that robust degradation takes place to remove *glp-1* mRNA after the action of Notch-Delta signaling between ABp and P2. Apart from these transcripts, P-granules seem to enrich mRNAs, as visualized by poly-A-tail probes (Class III, Figure 13C), but the nature of these mRNAs remains to be addressed. Recently, single cell mRNA deep sequencing revealed that 13 mRNAs are enriched in AB, while 4 transcripts are more abundant in P1 (Class IV, Figure 14 D and Table 1 on page 97; Hashimshony et al., 2012). Potentially interesting mRNAs are enriched in the AB cell, such as the mRNA of the anterior polarity gene *par-3*. It would be very interesting to probe if such anterior of *par-3* mRNA is important in regulating polarity. It is unknown whether the asymmetric distribution of these mRNAs is executed by asymmetric mRNA transport in the zygote and/or relying on differential degradation mechanisms acting in the two cell stage.

**Figure 14. Polarized mRNAs in early C. elegans embryos**
In conclusion, to date, non-uniform distribution of a given mRNA has not been reported in the one-cell stage embryo. To explore if such an mRNA may exist, we investigated the localization of the 11,237 mRNAs available in the publicly available in situ hybridization database (Nematode Expression Database (NEXTDB), http://nematode.lab.nig.ac.jp). In this database, the ESTs are listed on all chromosomes and by clicking on the EST one can retrieve the in situ hybridization image of early embryos and adult worms. This analysis confirmed that almost all transcripts displayed homogenous localization, indicating that the C. elegans one-cell stage embryo markedly differs from other systems, like the Drosophila embryo (Lecuyer et al., 2007).

However, Sachin Kotak uncovered a very striking localization pattern for the lin-5 mRNA. Moreover, I found that the mRNA of the uncharacterized gene W02F12.3 (dubbed era-1) is also non-uniformly distributed in the one-cell stage embryo. Exploring the mechanism and function of these two mRNA localization patterns constitutes the major topics of this thesis.

Furthermore, I also continued a project that was initiated by a former PhD student, Kalyani Thyagarajan, to investigate the role of clathrin heavy chain in pulling force generation in one cell stage embryos.
2. AIMS OF THE STUDY

• Characterization of the mechanisms and function of lin-5 mRNA localization
• Characterization of the mechanisms driving era-1 mRNA distribution
• Characterization of the effect of the clathrin cheavy chain on force generation
3. CHARACTERIZATION OF THE MECHANISMS AND FUNCTION OF LIN-5 mRNA LOCALIZATION

3A. RESULTS

*lin-5* mRNA is enriched around centrosomes in early *C. elegans* embryos

We explored whether the mRNAs encoding cortical force generator components may display an unusual localization by browsing the publicly available Nematode Expression Database. This analysis revealed that *lin-5* mRNA, as opposed to the mRNA of other members of the force generator complex, appears to exhibit a non-uniform localization in early embryos.

We decided to further investigate *lin-5* mRNA localization. As a first step, we set up the *in situ* hybridization method used for *C. elegans* embryos. Conducting conventional, digoxigenin- and alkaline-phosphatase (DIG-AP) based *in situ* hybridization experiments, we found that *lin-5* mRNA is enriched around centrosomes throughout the first cell cycle and displays an analogous localization in later stages of embryogenesis (Figure 15A-D). We noted that this enrichment becomes more pronounced during the first cell cycle as centrosomes mature.
**Figure 15.** *lin-5* mRNA is enriched around centrosomes in early *C. elegans* embryos

A-D Digoxigenin-AP *in situ* hybridization against *lin-5* mRNA in one-cell embryos at prophase (A), prometaphase (B), anaphase (C), as well as at the end of the two-cell stage (D). Schematics below show the distribution of the mRNA in different stages. Moreover, unless stated otherwise, all *in situ* hybridization experiments are conducted with digoxigenin, with mRNA shown in black and DNA in blue. Here and in other cases, n>50 embryos were analyzed and representative images are shown. In panel B and C, a black region from the original image resulting from image rotation was filled with grey dashed shapes.

We also implemented the single molecule FISH method (Raj et al., 2008) that confirmed enrichment of *lin-5* mRNA around centrosomes (Figure 16E-H). This method allows for more precise quantification of mRNA localization that we will take advantage of later on (see page 70-71).
Figure 16. Single molecule FISH confirms centrosomal enrichment of *lin-5* mRNA

A-D Single molecule FISH against *lin-5* mRNA at the same stages as in Figure 15A-D. *lin-5* mRNA is visualized with wide-field microscopy and shown in white. Small foci in the cytoplasm should correspond to single mRNA molecules.

The signal detected by the DIG-AP *in situ* hybridization is specific to *lin-5* mRNA since it is not observed in *lin-5(RNAi)* embryos (Figure 17C and D). By contrast to *lin-5* mRNA, *in situ* hybridization revealed a uniform cytoplasmic distribution for *goa-1* mRNA (Figure 17E and F). Moreover, when browsing the Nematode Expression Database, we did not observe similar localization for any of the other 11,237 mRNAs, indicating that such centrosomal localization is very rare, or even unique.

Figure 17. *lin-5* *in situ* hybridization signal is specific to *lin-5* mRNA and centrosomal enrichment is not observed for *goa-1* mRNA

A, B Localization of *lin-5* mRNA in wild type *C. elegans* embryos viewed at low (A) and high (B) magnification. The scale bar corresponds to 50 µm in the low and 10 µm in the high magnification views. C, D Localization of *lin-5* mRNA in *lin-5(RNAi)* *C. elegans* embryos
viewed at low (C) and high (D) magnification. E, F Localization of goa-1 mRNA in wild type C. elegans embryos viewed at low (E) and high (F) magnification.

As an initial step to evaluate the potential relevance of lin-5 mRNA localization, we addressed whether it is similarly enriched in the sister species C. briggsae, which is though to have diverged from C. elegans ~100 million years ago (Hillier et al., 2007). As shown in Figure 18A and B, we found this to be the case, supporting the notion that such localization may be functionally relevant.

**Figure 18. Caenorhabditis briggsae lin-5 mRNA is likewise enriched at centrosomes**
A, B Localization of lin-5 mRNA in C. briggsae viewed at low (A) and high magnification (B). The scale bar corresponds to 50 µm in the low and 10 µm in the high magnification view.

**Microtubules and dynein are necessary for lin-5 mRNA enrichment around centrosomes**

We set out to dissect the mechanisms underlying lin-5 mRNA enrichment around centrosomes. To test if centrosomes are sufficient, we analyzed zyg-12(RNAi) embryos, in which centrosomes detach from pronuclei and nuclei. As shown in Figure 19A, we found that lin-5 mRNA follows centrosome position in such embryos, demonstrating that centrosomes are sufficient to dictate localization. Moreover, we asked if centrosomes are necessary for lin-5 mRNA localization by examining spd-5(RNAi) embryos, in which centrosome assembly is impaired (Hamill et al., 2002). We found that lin-5 mRNA still exhibits focused enrichment around pronuclei and chromatin in these embryos (Figure 19B). Therefore, centrosomes are not essential for lin-5 mRNA localization. Since microtubules are still nucleated to some extent around pronuclei and chromatin in the absence of SPD-5 function (Hamill et al., 2002), we reasoned that lin-5 mRNA enrichment in spd-5(RNAi) embryos may be due to the presence of microtubules. To test this hypothesis, we interfered with microtubules by
using RNAi against the gene tbb-1 (Sonnichsen et al., 2005), and found that lin-5 mRNA distribution is uniform in this case (Figure 19C). Through which mechanism could microtubules be needed for lin-5 mRNA localization around centrosomes? The minus-end directed microtubule-dependent motor dynein seemed like a plausible candidate. Accordingly, we found that lin-5 mRNA is distributed uniformly in the cytoplasm in embryos depleted of the dynein heavy chain DHC-1 (Figure 19D). Overall, we conclude that dynein-dependent transport of lin-5 mRNA towards the minus-end of microtubules ensures lin-5 mRNA enrichment around centrosomes.

Figure 19. Microtubules and dynein drive lin-5 mRNA localization
A-D lin-5 mRNA localization in one-cell mitotic embryos depleted by RNAi of ZYG-12 (A), SPD-5 (B), TBB-1 (C) or DHC-1 (D). Cartoons below the embryos represent the status of centrosomes, microtubules, dynein and lin-5 mRNA in each condition. PCM: pericentriolar material.
The 3’ UTR is dispensable for lin-5 mRNA localization

We next sought to identify the cis-acting elements of the lin-5 mRNA that direct its centrosomal enrichment. Since in many mRNAs the 3’ untranslated region (3’ UTR) is responsible for subcellular localization (reviewed in Andreassi and Riccio, 2009), we tested the role of the lin-5 3’ UTR in mRNA centrosomal enrichment. To this end, we utilized a LIN-5-GFP strain in which the pie-1 promoter drives transcription of a chimeric mRNA comprising the protein coding region of the lin-5 cDNA fused to gfp and to heterologous 5’ and 3’ UTRs from the pie-1 gene (Figure 20C). Performing in situ hybridization against gfp mRNA, we found that lin-5-gfp mRNA is enriched around centrosomes in a manner analogous to endogenous lin-5 (compare Figure 20B with 20D). We also found that lin-5-gfp mRNA is enriched at centrosomes even in a condition in which the endogenous mRNA is mislocalized (Figure 20E, see later section for clarification of the root of the mislocalization caused by the ev571 mutation). This rules out the possibility that the transgene is localized simply because it hitchhikes on the endogenous mRNA.

Based on these experiments, we conclude that the lin-5 5’ UTR and 3’ UTR are dispensable, whereas the lin-5 coding region is sufficient, for enrichment of lin-5 mRNA around centrosomes.

**Figure 20. The 3’ UTR is dispensable, while the coding region is sufficient for lin-5 mRNA localization**

A Cartoon representing the linear structure of lin-5 mRNA. Exon 3 is marked with black dots, see explanation later for this. B Endogenous lin-5 mRNA localization. C Cartoon representing the linear structure of lin-5-gfp mRNA. D lin-5-gfp mRNA visualized with gfp probe. E lin-5-gfp mRNA visualized with gfp probe in the lin-5(ev571) mutant background.
The coding region is necessary for lin-5 mRNA localization

Next, we sought to identify the mRNA localization sequence within the coding region. To narrow down the position of such a signal, we took advantage of a functional lin-5 transgene cloned in a pie-1 promoter/pie-1 3’ UTR cassette that was recoded in the first 376 nucleotide (lin-5[rec376], Figure 21A; Galli et al., 2011). This construct is RNAi resistant against dsRNAs targeting the first 376 nucleotides, allowing one to perform RNAi to deplete endogenous lin-5. We hypothesized that, provided the localization sequence is within this first 376 nucleotide of the endogenous mRNA, recoding should interfere with centrosomal localization of the transgene. Performing in situ hybridization in the absence of endogenous lin-5, we observed centrosomal enrichment of this transcript (Figure 21B). This indicates that the localization signal is not located in the first 376 nucleotides, and further demonstrates that the 3’ UTR is dispensable for mRNA localization, even in the absence of the endogenous mRNA. Moreover, these data prove that the nucleotides between the positions 376 and 2466 are sufficient for localization.

Figure 21. The coding region is necessary for lin-5 mRNA localization

To test whether the entire lin-5 coding region is necessary for mRNA enrichment, we designed a lin-5 coding sequence that retains the normal amino acid sequence but contains altered nucleotides along the entire coding region. We inserted this recoded sequence (hereafter referred to as lin-5[rec]) in a pie-1 promoter/pie-1 5’ UTR/pie-1 3’UTR cassette (Figure 21C) and generated a transgenic line with this
construct. The expression level of the transgene was only ~13% that of endogenous lin-5 (Figure 22A), presumably owing to silencing of the transgenic constructs. Nevertheless, we were able to perform in situ hybridization against the lin-5[rec] transcript and found that lin-5[rec] is distributed uniformly in the cytoplasm (Figure 21C). Due to the low expression level of this transcript, we could not test the function of mislocalized lin-5 mRNA. Generation of new lines that might allow for such analysis is in progress.

Based on the above-mentioned results, we conclude that the lin-5 coding region is sufficient and also necessary for proper mRNA location.

Figure 22. Levels of lin-5[rec] mRNA
qPCR analysis of lin-5[rec] mRNA. Levels of lin-5[rec] is expressed relative to endogenous lin-5. n=3 experiments.

A 9-nucleotide insertion in the coding region interferes with lin-5 mRNA localization

We then wondered if extant lin-5 mutant alleles could help narrow down the sequence directing mRNA localization. The lin-5(ev571) temperature sensitive mutant allele harbors a 9-nucleotide insertion in the coding region that is thought to disrupt function by impairing its coil-coiled domain, giving rise to dead progeny at the restrictive temperature (Figure 25D, Lorson et al., 2000). Although it is reported that LIN-5 protein localization is unaffected in these embryos, careful investigation of immunofluorescence images revealed a slight, but significant drop of centrosomal LIN-5 level (Figure 23A-C). On the other hand, overall cortical levels are not affected in these embryos.
Figure 23. Centrosomal LIN-5 protein levels are reduced in lin-5(ev571) embryos
A-B LIN-5 protein localization at anaphase in wild type and in lin-5(ev571) embryos at the restrictive temperature. The upper images show the merge of DNA (blue), α-tubulin (green) and LIN-5 (red), the lower image displays LIN-5. C Quantification of centrosomal and cortical (both anterior and posterior) signal both relative to cytoplasmic values in wild type and lin-5(ev571) embryos. n=18 wild type and n=14 lin-5(ev571) embryos.

Intriguingly, we found that lin-5 mRNA is mislocalized in lin-5(ev571) embryos at the restrictive temperature (Figure 25F). This result suggests either that LIN-5 protein function and proper centrosomal LIN-5 levels are needed for correct localization of lin-5 mRNA or that the 9-nucleotide insertion disrupts the mRNA localization signal. As a step towards distinguishing between these possibilities, we analyzed aspm-1(RNAi) embryos, in which LIN-5 protein is absent from centrosomes (van der Voet et al., 2009). We confirmed the absence of spindle positioning phenotype of aspm-1(RNAi) embryos, indicating that the centrosomal pool of LIN-5 protein is not crucial for spindle positioning. More importantly, we found that lin-5 mRNA distribution is normal in embryos depleted of ASPM-1 (Figure 24), demonstrating that centrosomal LIN-5 protein is dispensable for lin-5 mRNA centrosomal enrichment.

Figure 24. Centrosomal LIN-5 protein is dispensable for lin-5 mRNA localization
A lin-5 mRNA localization in aspm-1(RNAi) embryos. Arrowhead points to evidence of meiotic defects.
Compatible with LIN-5 protein not being required elsewhere in the cell either, we found furthermore that lin-5 mRNA is also mislocalized in lin-5(ev571) embryos at the permissive temperature (data not shown). Next, we took advantage of a lin-5(ev571) revertant line, lin-5(ev571he63), which harbors a single nucleotide missense mutation 16 nucleotides upstream of the ev571 insertion site (Figure 25G; Fisk Green et al., 2004). This revertant exhibits a near-complete rescue of embryonic lethality at the restrictive temperature (Fisk Green et al., 2004), indicating that LIN-5 protein is functional, presumably through restoration of the coil-coiled domain (Fisk Green et al., 2004). Importantly, we found that lin-5 mRNA nevertheless remains distributed uniformly in lin-5(ev571he63) embryos (Figure 25I). This reinforces the notion that lin-5 mRNA can be mislocalized despite LIN-5 protein being functional and that the ev571 insertion impairs the localization signal. Overall, these results raise the possibility that the localization signal per se is disrupted by the ev571 insertion.
Figure 25. A 9-nucleotide insertion in the coding region interferes with lin-5 mRNA localization

A Schematics of wild type lin-5 exon 3 and the predicted structure of the coil-coiled domain translated from this region. The same holds for D and G. B Wild type embryo at the stage of centration/rotation showing no meiotic defect. C Endogenous lin-5 mRNA localization. Embryonic viability at 25°C is given below the images in C, F and I. D Schematics of exon 3 in lin-5(ev571). The red region represents the 9-nucleotide/3-amino acids insertion. E lin-5(ev571) embryo at restrictive temperature at the stage of centration/rotation displaying meiotic defects (arrowheads). Note that no meiotic defect is observed at the permissive temperature. F lin-5 mRNA distribution in lin-5(ev571) mutant embryo at the restrictive temperature. G Schematics of exon 3 in lin-5(ev571he63). The green region shows the single nucleotide reverting substitution. F lin-5(ev571he63) embryo at the stage of centration/rotation showing no meiotic defect. I lin-5 mRNA distribution in the lin-5(ev571he63) revertant.

We found wild type levels of total lin-5 mRNA and total LIN-5 protein in lin-5(ev571) and in lin-5(ev571he63) embryos (Figure 26A and B), indicating that mRNA mislocalization does not influence mRNA- and protein stability.

Figure 26. Embryos with mislocalized lin-5 mRNA exhibit wild-type level of lin-5 mRNA and LIN-5 protein

A qPCR analysis of lin-5 mRNA in wild type, lin-5(ev571) and in lin-5(ev571he63) embryos at permissive (black) and restrictive (grey) temperatures. Values are expressed relative to act-1 mRNA. n=1 experiment. B Western blot analysis of LIN-5 protein from embryonic lysates in wild type, lin-5(ev571) and in lin-5(ev571he63) embryos at the restrictive temperature. Asterisks mark bands that are sometimes detected by LIN-5 antibodies (Nguyen-Ngoc et al., 2008). α-tubulin was used as a loading control. n=2 experiments.

Prompted by these findings, we sought to identify conserved mRNA motifs in the vicinity of the region disrupted by the ev571 insertion in four related nematode species (C. elegans, C. briggsae, C. remanei and C. brenneri). Suggestively, we found a 15-nucleotide stretch spanning the ev571 insertion site that is highly conserved among these species (Figure 27A). This observation might indicate that
this 15-nucleotide stretch is somehow important for lin-5 mRNA localization around centrosomes. We sought to test if the region around the ev571 insertion site forms any stem loop structure, indicative for RNA-protein interaction. We utilized three RNA structure prediction softwares, Mfold, RNAstructure and RNAfold, and found that a 75-nt region around the ev571 site forms a secondary structure (Figure 27B-D). However, these secondary structures do not show any obvious similarities when comparing these species, perhaps because the 75-nt long region is not long enough to reveal structural similarities. On the other hand, this might indicate that the nucleotide sequence, rather than the secondary structure is important for mRNA localization. Further experiments, including ones in which the nucleotides in that region would be recoded, will be needed to address the function of this 15-nt stretch.

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**Figure 27.** An evolutionary conserved 15-nt region in the vicinity of the ev571 site forms secondary structures

A An evolutionary conserved 15-nucleotide stretch around the ev571 insertion site in *C. elegans*, *C. briggsae*, *C. remanei* and *C. brenneri* found by the MEME algorithm (Bailey et al., 2009). The starting position of this stretch in the cDNA sequence of each species is indicated below the first nucleotide. Bold letters show conserved region, normal ones variable ones. B-D RNA structure prediction of a 75-nt long region around the ev571 site (30 nucleotides 5’ and 3’ from the 15-nt conserved region) with the softwares Mfold (B), RNAstructure (C) and RNAfold (D). Red line indicates the position of the 15-nt stretch; green star marks the starting point of the 75-nt region.
**lin-5 mRNA mislocalization correlates with decreased posterior pulling forces and with misdistribution of the force generator complex**

To test the relevance of *lin-5* mRNA localization, we took advantage of *lin-5(ev571)* embryos at the permissive temperature and *lin-5(ev571he63)* revertant embryos at the restrictive temperature. In both cases, *lin-5* mRNA is mislocalized despite what appears to be functional LIN-5 protein. Importantly, although *lin-5(ev571he63)* mutants exhibit only ~4% embryonic lethality (Fisk Green et al., 2004), analysis by time-lapse DIC microscopy enabled us to uncover fully penetrant spindle positioning defects in one-cell embryos. In particular, the robust oscillations of the posterior spindle pole during anaphase characteristic of the wild-type (Figure 28A) are not observed in *lin-5(ev571he63)* embryos (Figure 28D). To estimate the extent of pulling forces acting on spindle poles, we performed spindle severing experiments on these embryos. In line with the presence of decreased posterior spindle oscillations, we revealed a significant decrease in net pulling forces on the posterior side (Figure 28E). These experiments further support the notion that LIN-5 protein function is restored in these revertants, since anterior peak velocities are indistinguishable from the wild type. Importantly, posterior spindle pole oscillations are also diminished in *lin-5(ev571)* embryos at the permissive temperature (Figure 28B), further demonstrating that mislocalized *lin-5* mRNA correlates with a diminished pulling forces acting on the posterior side. Importantly, we cannot exclude that the protein is not fully functional in these backgrounds. Meiotic defects that are prevalent in embryos where LIN-5 function is perturbed are not observed in *lin-5(ev571)* embryos at the permissive temperature or in *lin-5(ev571he63)* embryos at the restrictive temperature (Figure 25B, E and H), suggesting that, as far as meiosis in concerned, LIN-5 protein regained function in the revertant strain. Note, however that partial reduction of GPR-1 leads to a dramatic drop in the amplitude of posterior spindle pole oscillation (Pecreaux et al., 2006). Therefore, we cannot rule out the possibility that different thresholds of functional LIN-5 protein operate for proper spindle pole oscillation and pulling forces, and the phenotypes observed are the consequence of not fully functional LIN-5 protein.
Figure 28. Mislocalization of lin-5 mRNA results in decreased posterior pulling forces
A-D Representative anterior (green) and posterior (blue) spindle pole oscillations in wild type (A), lin-5(ev571) at permissive (B) and restrictive temperature (C) or lin-5(ev571he63) (D). Tracking of spindle poles was performed from the onset of posterior spindle displacement until cytokinesis onset. E Spindle severing experiments revealing net pulling forces acting on spindle poles at anaphase. Number of embryos analyzed: wild type (n=7), lin-5(ev571) (n=5) and lin-5(ev571he63) (n=8).

Having this possibly caveat in mind, we decided to investigate lin-5(ev571he63) embryos. As an other means to investigate the consequence of the decrease in pulling forces on the posterior side, we challenged lin-5(ev571he63) embryos with goa-1(RNAi), in which spindle positioning is slightly impaired. We found that goa-1(RNAi) results in ~25% embryonic lethality in the wild type, but in ~80% embryonic lethality in the lin-5(ev571,he63) background (Figure 29), suggesting that pulling forces are impaired in the revertant.

Figure 29. Mislocalization of lin-5 mRNA sensitizes the embryos to slight malfunction of the force generator complex
Embryonic lethality in wild type and lin-5(ev571he63) embryos upon goa-1(RNAi). n>100 embryos were analyzed in each case.
To investigate the cause of decreased posterior pulling forces in \textit{lin-5}(ev571he63), we performed immunofluorescence analysis to reveal the distribution of cortical force generator components in \textit{lin-5}(ev571) embryos at the permissive temperature and in \textit{lin-5}(ev571he63) embryos at the restrictive temperature. As GPR-1/2 exhibits the most obvious asymmetry between the anterior and posterior cortices in the wild-type, we focused our analysis on this component. We found that whereas GPR-1/2 is slightly enriched on the posterior cortex during mitosis in the wild-type (Figure 30A, quantified in D), this slight asymmetry is absent at the restrictive temperature in \textit{lin-5}(ev571) and \textit{lin-5}(ev571he63) embryos (Figure 30B and C, quantified in D). These findings establish that there is less GPR-1/2 at the posterior cortex in these conditions than there is in the wild-type, which likely explains the observed decrease in net forces pulling on the posterior side.

Taken together, these results indicate that \textit{lin-5} mRNA enrichment around centrosomes is important for proper generation of pulling forces acting on the posterior spindle pole in one-cell \textit{C. elegans} embryos.

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\caption{Mislocalization of \textit{lin-5} mRNA leads to misdistribution of the force generator complex}
\end{figure}
A-C GPR-1/2 immunostaining of wild type (A), lin-5(ev571) (B) and lin-5(ev571he63) (C) anaphase embryos at restrictive temperature. The upper images show the merge of DNA (blue), α-tubulin (green) and GPR-1/2 (red), the lower images the GPR-1/2 signal alone. The arrowhead in B points to meiotic defects. D Quantification of cortical GPR-1/2 enrichment relative to the cytoplasm in embryos of indicated genotypes. Insets display magnified region of anterior (green) and posterior (blue) cortex. Number of embryos analyzed: wild type (n=9), lin-5(ev571) (n=10) and lin-5(ev571he63) (n=9).

*lin-5 mRNA centrosomal enrichment is asymmetric in anaphase*

How could centrosomal localization of *lin-5* mRNA promote the asymmetric distribution of cortical force generators? To begin addressing this question, we quantified *lin-5* mRNA enrichment around the two centrosomes through the first cell cycle using single molecule FISH (Figure 31). Interestingly, this analysis revealed that *lin-5* mRNA enrichment at the anterior centrosome increases as the cell progresses from prometaphase to anaphase, while the posterior signal is unchanged. Such unequal enrichment leads to asymmetric enrichment at anaphase between the two centrosomes, with significantly less *lin-5* mRNA present around the posterior centrosome compared to the anterior one (Figure 31).

![Figure 31. *lin-5* mRNA enrichment increases at the anterior spindle pole during the first cell cycle.](image)

Centrosomal enrichment relative to cytoplasmic signal were quantified in prometaphase, metaphase and anaphase embryos. n= 13, 8 and 14, respectively.

We next tested if such centrosomal asymmetry in anaphase depends on A-P polarity cues by examining *lin-5* mRNA localization in embryos lacking the function of the anterior PAR protein PAR-3 or the posterior PAR protein PAR-2. Importantly, we found that *par-3(it74)* embryos or *par-2(RNAi)* embryos exhibit no difference in the extent of *lin-5* mRNA enrichment around the two centrosomes (Figure 32B-C, and
We conclude that A-P polarity cues regulate the asymmetric enrichment of lin-5 mRNA around centrosomes in anaphase.

The above experiments revealed that lin-5 mRNA enrichment at centrosomes is symmetric when pulling forces acting on spindle poles are either low or high on both sides. To address if unequal forces are indeed important for the asymmetric enrichment of lin-5 mRNA around the two centrosomes, beyond conditions in which A-P polarity is perturbed, we examined embryos with normal A-P polarity but altered pulling forces. We found that embryos in which forces are higher on both sides due to expression of YFP-GPR-1 exhibit more similar lin-5 mRNA enrichment around both anterior and posterior spindle poles (Figure 32D and I). Similarly, upon depletion of GPR-1/2 when pulling forces are equally weak on both sides, the extent of lin-5 mRNA enrichment at the two spindle poles is equal (Figure 32E and J). Overall, these findings establish that unequal net pulling forces contribute to asymmetric lin-5 mRNA enrichment at centrosomes, suggesting a positive feedback mechanism whereby increased pulling forces on the posterior side contribute to lowering the amount of lin-5 mRNA around the posterior spindle pole (see Discussion).

Although we have not performed quantitative single molecule FISH in C. briggsae, the difference of lin-5 mRNA signal between the two spindle poles at anaphase is seemingly also present in this species (see Figure 18B), suggesting that this feature is evolutionary conserved and thus of biological relevance.
Asymmetric enrichment of lin-5 mRNA at anaphase is polarity- and force-dependent

A-E Z projection of confocal sections of lin-5 mRNA single molecular FISH probes in wild type (A), par-3(it74) (B), par-2(RNAi) (C), YFP-GPR-1 (D) or gpr-1/2(RNAi) (E) anaphase embryos. Cartoons on the right represent the status of polarity (green: anterior, blue: posterior), of forces acting on the two spindle poles (green and blue arrows, respectively) and of lin-5 mRNA centrosomal enrichment in each condition. F-J Quantification of lin-5 mRNA centrosomal enrichment (relative to cytoplasm) in the conditions described in A-E. Numbers of embryos analyzed: wild-type (n=16), par-3(it74) (n=9), par-2(RNAi) (n=8), YFP-GPR-1 (n=10), gpr-1/2(RNAi) (n=8).

de novo lin-5 translation/folding occurs preferentially in the cytoplasm during mitosis

To explore the potential importance of having less lin-5 mRNA around the posterior centrosome, we addressed if lin-5 mRNA is translated during mitosis using
embryos expressing LIN-5-GFP. Cortical LIN-5-GFP is not detectable in one-cell embryos of this strain, and we focused our analysis on the centrosomal and cytoplasmic signals (Figure 34A-E). To address whether lin-5 mRNA is translated during mitosis, we developed an assay that we dubbed Fluorescence Recovery After complete Photobleaching (FRAcP). The goal of this assay is to completely bleach the LIN-5-GFP signal in the embryo and record fluorescence intensity thereafter to investigate the extent of de novo protein synthesis and protein folding (Figure 33A). If no fluorescence recovery is observed, the conclusion would be that no protein is translated/folded de novo (Figure 33B, upper most scenario). By contrast, if fluorescence signal recovery is observed, this could reflect de novo protein synthesis or the folding of already translated LIN-5-GFP.

Figure 33. Principle of the FRAcP experiment and its possible interpretations
A Schematic illustration of the Fluorescent Recovery after Complete Photobleaching (FRAcP) experiment. B Possible outcomes of FRAcP experiments with corresponding conclusions.

As shown in Figure 34F-K, FRAcP experiments revealed that LIN-5-GFP fluorescence does recover after complete bleach to ~17% at centrosomes and to ~22% in the cytoplasm of the initial intensities measured before the bleach. Therefore, LIN-5-GFP undergoes folding and/or translation during mitosis in one-cell C. elegans embryos. We attempted to block translation by treating the embryos with the translational inhibitor cycloheximide (CHX). However, we found no major phenotypic effect, such as cell cycle arrest, suggesting that the concentration of CHX used in these experiments (10mg/ml) is not high enough or that the drug cannot act within the time frame of the experiment. Thus, not having discriminated between the two processes, we will refer to the observed recovery as translation/folding.
We reasoned that FRAcP might also reveal whether *lin-5* mRNA translation/folding takes place preferentially at centrosomes or in the cytoplasm (Figure 33B). Given that *lin-5* mRNA exhibits a ~3 fold enrichment at centrosomes compared to the cytoplasm, a similar 3:1 centrosomal versus cytoplasmic ratio is expected for LIN-5-GFP signal recovery if translation/folding takes place with similar efficiency regardless of subcellular localization. By contrast, a ratio higher than 3:1 would suggest preferential translation/folding of the centrosomal pool. Conversely, a ratio smaller than 3:1 would suggest preferential translation/folding of the cytoplasmic pool. As shown in Figure 34L, we found that GFP recovery is observed initially in the cytoplasm and that the centrosomal versus cytoplasmic ratio of the fluorescence intensities is ~1. Therefore, *lin-5* mRNA is translated/folded preferentially in the cytoplasm.

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**Figure 34. LIN-5 is de novo translated/folded during mitosis**
A-E Images from a time-lapse recording of one-cell embryo expressing LIN-5-GFP. Insets show magnified region of the cytoplasm (red) and the centrosome (green) at prophase (A), prometaphase (B), metaphase (C), anaphase (D) and telophase (E). t=0 corresponds to cytokinesis onset. F-O Complete FRAP (cFRAP) in LIN-5-GFP embryo; insets are as in A-E. F Pre-bleach fluorescence intensities. G Post-bleach fluorescence intensities. H-J Fluorescence recovery at metaphase (H), anaphase (I) and telophase (J). K Cytoplasmic (red) and centrosomal (green) signal recovery relative to pre-bleach values (black square) plotted as a function of time. Intensities in the cytoplasm and at centrosomes are shown relative to pre-bleach!
bleach signals. n=8 embryos. L Centrosomal/cytoplasmic signal intensities during mitosis. n=8.

Next, in order to address if LIN-5-GFP molecules shuttle between the cytoplasm and centrosomes, we performed two types of Fluorescence Loss In Photobleaching (FLIP) experiments (Figure 35A). We either bleached extensively the anterior centrosome and measured fluorescence intensities in the neighboring cytoplasm (referred to as ‘centro-bleach’) or bleached a cytoplasmic region and then quantified fluorescence intensities of the centrosome (‘cyto-bleach’). We found decreased intensities of the unbleached region in both cases (Figure 35B), showing that LIN-5-GFP molecules are transported bi-directionally during mitosis. Quantifying the extent of fluorescence decrease in the unbleached regions revealed that the centrosomal pool decreases to a greater extent upon cyto-bleach than does the cytoplasmic pool upon centro-bleach (38% vs. 19%, Figure 35C). This is not the consequence of unequal bleach, because the two post-bleach values were comparable. Given that the LIN-5-GFP signal and thus supposedly the number of molecules are ~2-fold higher at centrosomes at the time of the bleach as compared to the cytoplasm, we likely bleach 2 times more molecules during the centro-bleach than during the cyto-bleach. Based on this, we speculate that the bias in the direction of the transport is underestimated by this method and is higher in reality. These results, together with the FRACP data, suggest that LIN-5-GFP molecules synthetized/folded de novo in the cytoplasm move to the centrosome, explaining how LIN-5 protein can accumulate at that location despite preferential translation in the cytoplasm.

Taken together, these experiments suggest that lin-5 mRNA is translated/folded preferentially in the cytoplasm during mitosis.
Figure 35. Bi-directional transport of LIN-5-GFP is favored from cytoplasm to centrosome than from centrosome to cytoplasm during mitosis
A Schematics explaining two versions of Fluorescence loss after photobleaching (FLIP) experiments performed in LIN-5-GFP embryos. Either the centrosome was bleached and the intensities in the cytoplasm were measured (centro-bleach), or the cytoplasm was bleached and the centrosomal intensity was registered (cyto-bleach). B Relative signal intensities measured for untreated, centro-bleach and cyto-bleach conditions. Fluorescence intensities at centrosomes (green) and in the cytoplasm (red) were measured at telophase and plotted relative to the metaphase values. n=6 embryos. C Fold decrease of centrosomes (green) and cytoplasm (red) after centro- or cyto-bleach.

Morpholino-mediated inhibition of lin-5 mRNA translation leads to decreased posterior pulling forces

We sought to test the importance of de novo synthesized LIN-5 protein by inhibiting lin-5 mRNA translation using morpholinos (MO). MOs efficiently block protein synthesis by binding to the translational initiation site of mRNAs and have been widely used in model systems such as Xenopus or zebrafish as well as in other nematode species (Louvet-Vallee et al., 2003). However, to our knowledge, MOs have never been utilized in C. elegans. To evaluate the potency of MOs in C. elegans, we injected lin-5 MOs in the gonad and found that the majority of the resulting embryos exhibit a strong lin-5 phenotype, included pronounced meiotic defects (Figure 36D). For the exact quantification of this phenotype, see the Materials and Methods section on page 136.

Figure 36. lin-5 morpholinos are capable of inhibiting lin-5 translation in the gonad
A-D: One-cell embryos during centration/rotation: wild type (A), lin-5(RNAi) (B), control MO injected (C) and lin-5 MO injected (D). Arrowheads indicate the presence of aberrant polar bodies and multiple female pronuclei, which are both hallmarks of meiotic defects.
Next, to block \textit{lin}-5 translation specifically in the first cell cycle, we reasoned that MOs coated with lipofectamine (LF) could enter the embryo after piercing the eggshell with a laser beam, as done previously for drug treatments (Figure 37A). After piercing the eggshell, we investigated the impact of interfering with \textit{lin}-5 translation during mitosis on anaphase pulling forces. Strikingly, we found that whereas embryos treated with control MOs exhibit normal oscillations (Figure 35B), impairing \textit{lin}-5 translation results in decreased oscillations of the posterior spindle pole (Figure 37C). Moreover, spindle severing experiments uncovered a significant decrease in pulling forces specifically on the posterior spindle pole (Figure 37D). These findings demonstrate that \textit{lin}-5 mRNA translation during mitosis contributes to generating proper pulling forces.

![Diagram](image)

**Figure 37. Impairing translation of \textit{lin}-5 mRNA during mitosis results in decreased posterior pulling forces**

A Schematic representation of morpholino experiment. Wild type embryos were used for DIC and for spindle severing experiments. The eggshell was pierced with a UV-laser at the pseudocleavage stage to let the lipofectamine-coated morpholino molecules enter the cell. B-C Spindle pole oscillations of representative wild type embryo treated with control (B) or \textit{lin}-5 (C) MOs. Anterior (green) and posterior (blue) spindle pole were tracked. Tracking of spindle poles was performed from the onset of posterior spindle displacement until
cytokinesis onset. D Spindle severing experiments performed in embryos treated with control or *lin-5* MOs. Number of embryos analyzed: control MO (n=14), *lin-5* MO (n=15) from 3 experiments.
3B. DISCUSSION

We uncovered here that lin-5 mRNA is enriched around centrosomes during early *C. elegans* development. We demonstrated that this enrichment is less pronounced around the posterior spindle pole than around the anterior spindle pole in anaphase one-cell embryos. We also established that embryos in which lin-5 mRNA is distributed in a uniform manner exhibit reduced pulling forces on the posterior spindle pole. Combined with the fact that translation/folding of LIN-5-GFP occurs more readily in the cytoplasm than at centrosomes, these findings lead us to propose a working model that is summarized in Figure 38. In this model, following the capture of the mRNA at the anterior centrosome in anaphase, more lin-5 mRNA is available for translation in the posterior cytoplasm. As a result, slightly more LIN-5 protein is available on the posterior side, which contributes to generating a larger net pulling force on the posterior spindle pole. Even though the trigger for the capture of lin-5 mRNA from at the posterior centrosome remains to be identified, we postulate that more lin-5 mRNA in the cytoplasm can lead to increased LIN-5 production and increased pulling forces on the posterior side, thus creating a positive feedback loop. That this is the case is underscored by the finding that modulating pulling forces by altering GPR-1/2 levels impacts on lin-5 mRNA localization. Importantly in addition, we found that seemingly different levels of lin-5 mRNA on the two centrosomes are also present in *C. briggsae* embryos during mitosis, demonstrating that asymmetric lin-5 mRNA localization at anaphase is evolutionary conserved.
mRNA enrichment around centrosomes: beyond lin-5

There is ample precedent in other systems for mRNAs being enriched around centrosomes. For instance, many mRNAs, including ones encoding putative developmental transcription factors, are enriched around centrosomes in embryonic blastomeres of the spiralian lophotrochozoan gastropod *Crepidula fornicata* (Henry et al., 2010). Similarly, mRNAs are enriched at centrosomes in the mollusk *Spisula solidissima* (Alliegro and Alliegro, 2008; Alliegro et al., 2006). Likewise, numerous transcripts localize to the Centrosome Attracting Body (CAB), an organelle associated with one of the two centrosomes in ascidian embryos (reviewed in Prodon et al., 2007). As a result, these mRNAs are inherited by only one of the two daughter cells following mitosis. Similarly, centrosomal mRNA enrichment ensures asymmetric segregation of transcripts crucial for developmental patterning and in cell fate.
specification in the mollusk *Illyana obsolete* (Chan and Lambert, 2011; Kingsley et al., 2007; Lambert and Nagy, 2002; Rabinowitz et al., 2008; Rabinowitz and Lambert, 2010; Swartz et al., 2008). The asymmetric inheritance is achieved by the transport of these mRNAs in a microtubule-dependent manner from the centrosome to the cortex where they are anchored by the actin cytoskeleton (Lambert and Nagy, 2002). Our findings in *C. elegans* indicate by contrast that the overall amount of *lin-5* mRNA is similar in the two daughters of the one-cell embryo. Accordingly, *lin-5* is not in the list of mRNAs differentially expressed between isolated AB and P<sub>1</sub> blastomeres (Hashimshony et al., 2012).

Is the enrichment around centrosomes conserved amongst mRNAs encoding LIN-5-related molecules across evolution? We showed that such localization is also observed in *C. briggsae*, indicating conservation for at least ~100 million years. We note, however, that the mRNA encoding the LIN-5 related protein NuMA is not amongst the microtubule-associated transcripts that have been identified in *Xenopus* egg extracts, indicating that centrosomal enrichment might not extend to vertebrates (Blower et al., 2007). The situation may differ in *Drosophila*, where examination of a publicly available *in situ* hybridization resource reveals that the mRNA encoding the LIN-5-related protein Mud exhibits an intriguing perinuclear focal localization compatible with centrosomal enrichment (see Figure 39 and http://fly-fish.ccbr.utoronto.ca/insitu_image_storage/img_dir_43/insitu43389.jpeg; DNA in red, *mud* mRNA in green (Lecuyer et al., 2007). Therefore, it may be that the enrichment around centrosomes uncovered in this study will prove significant in other systems.

Figure 39. *mud* mRNA appears to form perinuclear punctae resembling centrosomes in *Drosophila* embryos.
Low (A) and high magnification (B) of an mRNA probed for *mud* mRNA (green) and stained for DNA (red). Scale bars are 100 and 20 microns. Image source: Lecuyer et al., 2007.
Localization as a means to control translation of lin-5 mRNA

Translational repression is a widespread mechanism regulating localized mRNAs, which can act at different steps of the translational process. Thus, the Fragile X Mental retardation protein (FMRP) RNA binding protein recruits the eIF4E binding protein CYFIP1 to target mRNAs as they are transported from neuronal cell bodies to dendrites, thereby interfering with translation initiation (Napoli et al., 2008). Furthermore, as mentioned in the Introduction, complex mechanisms ensure translational regulation of the ash1 mRNA in budding yeast or of oskar, bicoid, nanos and gurken mRNAs in the Drosophila oocyte (see Introduction on pages 42-44). Translational repression can occur via inhibition of the 40S and the 60S ribosomal subunit assembly (in the case of ash1 mRNA) or by interfering with the elongation factor 4E (for oskar and nanos mRNAs). It will be interesting to investigate at which step lin-5 mRNA translation is repressed at centrosomes.

In other systems, mislocalization of mRNAs can result in premature and thus excess translation, as for ash1 in budding yeast (Gu et al., 2004; Paquin et al., 2007) or oskar in Drosophila (Kim-Ha et al., 1995; Nakamura et al., 2004). By contrast, our data indicate that mis-localization of lin-5 mRNA to the cytoplasm is not sufficient for excess translation. This suggests that, regardless of the mechanism, another regulatory step, perhaps coupled to cell cycle progression, must take place to render lin-5 mRNA ready to be translated/folded in the cytoplasm. Interestingly, the phosphorylation status and thereby the membrane-binding capability of the LIN-5 homologue NuMA is regulated at the metaphase to anaphase transition by CDK1 in human cells (Kotak et al., 2013). It is intriguing to speculate that lin-5 mRNA translation is under the control of similar mechanisms.

We revealed that GPR-1/2 asymmetry in anaphase is lost when lin-5 mRNA is mislocalized, and we speculate that GPR-1/2 immunostaining is a reliable proxy for probing the overall distribution of force generator complexes. As far as LIN-5 is concerned, we found that overall cortical levels of LIN-5 are unchanged in lin-5(ev571) embryos (Figure 23), indicating that LIN-5 protein localization is seemingly independent of lin-5 mRNA localization. How about the asymmetry in LIN-5 cortical enrichment? LIN-5 asymmetry between the anterior and posterior cortex at anaphase is marginal, as only half of the embryos show more posterior enrichment at the cortex
compared to the anterior one (see Introduction on page 25; Park and Rose, 2008). Therefore, addressing this important question is experimentally difficult.

**lin-5 mRNA localization, preferential translation and asymmetric pulling forces**

Although this study represents the first report regarding the importance of translational regulation in one-cell *C. elegans* embryos, translational regulation has been established in other instances during early *C. elegans* development (see Introduction on pages 48-51). In all of these cases, orchestrated action of translational repressors and activators lead to localized protein translation. By analogy, at the subcellular level, perhaps preferential translation of *lin*-5 mRNA in the cytoplasm is due to the presence of a translational repressor at centrosomes or of a translational activator away from them.

Is it possible that translational repressors, which may act specifically on *lin*-5 mRNA or on other mRNAs as well, are enriched around centrosomes in *C. elegans* embryos? The existence of such factors is plausible, especially given that in human tissue culture cells GW/P bodies marking microRNA-mediated translational repression events (Jakymiw et al., 2005; Liu et al., 2005a; Liu et al., 2005b) are enriched around centrosomes (Aizer et al., 2008; Moser et al., 2011). In fact, there is precedent for differential translation of mRNAs enriched around centrosomes, except that protein synthesis occurs preferentially at centrosomes in those cases, in contrast to the situation with *lin*-5 mRNA. Thus, *Xenopus* Cyclin B1 and Xbub mRNAs are enriched at centrosomes, where they are translated preferentially (Groisman et al., 2000). Similarly, also in *Xenopus*, local translation on the mitotic spindle was postulated for mRNAs encoding components regulating chromosome segregation (Eliscovich et al., 2008).

Which proteins could regulate the translation of *lin*-5 mRNA? Two RNA-binding proteins acting in the zygote, SPN-2 and SPN-4, are potential candidates. Loss of SPN-2 causes a spindle alignment phenotype that is rescued by the depletion of the katanin protein, MEI-1 (Li et al., 2009). However, whether pulling forces acting on posterior pole is also rescued by reducing MEI-1 levels was not investigated, and could be potentially interesting from the point of view of *lin*-5 mRNA translation and LIN-5/GPR-1/2 distribution. Interestingly, loss of SPN-4
function results in the mitotic spindle in P1 being misoriented although the exact mechanism remains elusive (Gomes et al., 2001). Furthermore, a genome wide RNAi screen revealed that spn-4 depletion leads to decreased posterior spindle pole oscillation in 8/10 embryos (Sonichsen et al., 2005). It is interesting to speculate that SPN-4 positively regulates lin-5 mRNA translation thereby affecting spindle oscillation and spindle positioning. Recently, it was shown that in human cells, Casein Kinase ιε and δ isoforms are involved in the maturation of the 40S ribosomal subunit and that inhibition of the ε isoform interferes with mRNA translation by compromising eIF4E (Shin et al., 2014; Zemp et al., 2014). It is possible that CSNK-1 likewise effects general translation and thereby maybe LIN-5 protein distribution in C. elegans one-cell stage embryos.

We found that dynein and microtubules dictate centrosomal lin-5 mRNA localization. Dynein-dependent transport localizes numerous mRNAs in other systems where adaptor proteins link the mRNAs with the dynein complex (see Introduction on page 42 and 45). It is tempting to speculate that such adaptor proteins also take part in localizing lin-5 mRNA to the centrosome in C. elegans, and that their relocalization to the cytoplasm may be the initial trigger leading to the relocalization of lin-5 mRNA away from the posterior pole during anaphase.

Regardless of the mechanism at play in C. elegans one-cell embryos, our finding that lin-5 mRNA is translated/folded preferentially in the cytoplasm, coupled with the fact that there is less lin-5 mRNA around the posterior centrosome compared to the anterior one during anaphase, prompted us to test the importance of lin-5 protein synthesis during mitosis. To this end, we developed a time-resolved MO-based method to prevent lin-5 mRNA translation specifically in one-cell embryos. Although MOs have been widely used in model systems such as Xenopus or zebrafish to interrogate gene function, they have not been used in C. elegans prior to this work, nor have they been employed to interfere in a time-resolved manner with protein synthesis. These experiments enabled us to demonstrate that translation of lin-5 mRNA during mitosis is critical for achieving strong net pulling forces on the posterior spindle pole, while being dispensable for those acting on the anterior side. There is precedent for such rapid translational action in the case of retinal axonal growth cones in Xenopus. The transport and translation of β-actin mRNA is triggered by an external gradient of the chemotropic Netrin-1 that leads to polarized β-actin
translation within 5 minutes. As a result, the axon turns toward the source of the gradient (Leung et al., 2006; Yao et al., 2006).

In conclusion, these findings lead us to propose that the remarkable localization of lin-5 mRNA around centrosomes might be important for ensuring a robust asymmetry in pulling forces during the first division of C. elegans embryos by inhibiting lin-5 mRNA translation on the anterior side (Figure 38).
4. CHARACTERIZATION OF THE MECHANISMS DRIVING ERA-1 mRNA DISTRIBUTION

4A RESULTS

The mRNA of the W02F12.3 gene is enriched on the anterior side of P₀ and is inherited by the anterior lineage

Prompted by the intriguing localization of *lin-5* mRNA, we decided to browse through the entire *in situ* hybridization database with the aim of identifying other interesting localization patterns. Surprisingly, we found only a few non-homogenous distributions amongst all ~11.237 ESTs analyzed. Among these, the transcript of an uncharacterized gene, W02F12.3, showed the most striking localization pattern: the mRNA displayed a very clear bias towards the anterior side of the zygote and this anterior distribution was seemingly maintained in later embryonic stages as well. We dubbed this gene *era-1* (embryonic mRNA anterior) and decided to investigate it further.

We confirmed the asymmetric distribution of *era-1* mRNA by digoxigenin-alkaline phosphatase based *in situ* hybridization (Figure 40A-D). We found that the asymmetric enrichment of *era-1* mRNA becomes apparent at the stage of pronuclear meeting and persists thereafter in the zygote. Moreover, *era-1* mRNA is significantly enriched in the anterior blastomere AB compared to the posterior blastomere P₁ in two cell-stage embryos (Figure 41E), a difference that is also apparent when comparing the AB daughters ABa and ABp with the P₁ daughters EMS and P₂ (Figure 40D). We detected no signal in *era-1*(RNAi) embryos, indicating probe specificity (Figure 41B and 41E).
Figure 40. *era-1* mRNA is enriched in the anterior side of the zygote and in anterior blastomeres

A-D *era-1* mRNA localization in P₀ prophase (A), P₀ prometaphase (B), 2-cell stage (C) and 4-cell stage embryos. Here and in the following figures, the mRNA signal appears dark grey; the DNA is shown in blue. The schematics below the embryos illustrate where the mRNA is distributed. Scale bar here and in the following figures correspond to 10 microns.

**era-1** mRNA localization is driven by MEX-5 and its 3'UTR

We sought to address what regulates the remarkable localization of the *era-1* mRNA. First we asked if the anterior enrichment is polarity-dependent. To address this question, we analyzed *era-1* mRNA distribution in *par-3(it71)* mutant embryos and found uniform transcript localization in this case (Figure 41C, 41E). We conclude that polarity mediates the asymmetric localization of *era-1* mRNA.
In addition to polarity, we revealed that era-1 mRNA asymmetry is disrupted in embryos depleted of the anteriorly localized RNA-binding protein MEX-5 (Schubert et al., 2000, Figure 41D, quantified in 41E). Even though we found that polarity establishment is slightly impaired in embryos mex-5(RNAi) embryos (Figure 42), we hypothesize that MEX-5 does not regulate era-1 mRNA localization through such a slight malfunction of polarity but in a more direct manner (see below).

**Figure 42.** mex-5(RNAi) leads to a slight delay in polarity establishment
Size of endogenous PAR-2 domain evaluated by immunofluorescence in embryos at the stage of centration/rotation with or without mex-5 depletion. n= 13 (control), n=16 (mex-5(RNAi)).
To identify the cis-acting element driving the localization of era-1 mRNA, we fused the sequence coding for the yellow fluorescent protein (YFP) to the era-1 genomic sequence (exons, introns and 3'UTR) in an expression vector driven by the pie-1 promoter and the pie-1 5'UTR (yielding the yfp-era-1[e3'] construct, Figure 43A). We generated a transgenic line expressing this construct and performed in situ hybridization against yfp mRNA. We found that the yfp-era-1[e3'] mRNA is enriched in the anterior of the zygote and is present in anterior blastomeres thereafter (Figure 43B, quantified in 43G). This enrichment is less pronounced than that of the endogenous era-1 mRNA (compare Figure 41A and 43B), suggesting that the era-1 5'UTR also contributes to mRNA distribution or that the presence of the yfp sequence interferes slightly with the localization signal.

Sequences in the 3'UTR are responsible for the localization of a variety of mRNAs (Merritt et al., 2008). To test the contribution of the era-1 3'UTR in mRNA localization, we generated a chimeric transgenic line expressing yfp fused directly to the era-1 3'UTR (yielding the yfp[e3'], construct, Figure 43C). As shown in Figure 43D, our analysis uncovered a significant anterior bias of yfp[e3'], mRNA, although the extent of enrichment in AB versus P₁ is much less pronounced than with the endogenous transcript or with yfp-era-1[e3'] mRNA (compare Figure 43D with 41A and 43B). We conclude that the era-1 3'UTR, while not being sufficient for, contributes to the anterior localization of the mRNA.

To test if the era-1 3'UTR is necessary for anterior mRNA localization, we modified the yfp-era-1[e3'] construct by replacing the era-1 3'UTR with the pie-1 3'UTR (yielding the yfp-era-1[p3'] construct, Figure 43E), and generated a corresponding transgenic line. Strikingly, we found that the yfp-era-1[p3'] mRNA localizes in a homogenous manner, with no difference between the anterior and posterior blastomeres (Figure 43F, quantified in 43G). Therefore, the 3'UTR of era-1 is necessary for anterior mRNA localization.
YFP-ERA-1 protein is enriched in anterior blastomeres and is negatively regulated by the era-1 3'UTR

To address whether the anterior enrichment of era-1 mRNA is accompanied by a likewise distribution of protein, we analyzed live embryos derived from the three transgenic strains described above using dual differential interference contrast (DIC) and fluorescent time-lapse microscopy. This revealed that YFP-ERA-1[e3’] protein can be detected starting from the ~10 cell stage and is present solely in descendants of the anterior blastomere AB (Figure 44).
Figure 44. YFP-ERA-1 distribution during embryogenesis
Snapshots of an embryo expressing YFP-ERA-1[e3'] imaged with dual DIC and fluorescence time-lapse microscopy. DIC image is on the left; YFP signal is on the right. Here and in Figures 47 and 48, t=0 corresponds to cytokinesis onset of P0. Arrowhead points to YFP signal detected at cell-cell boundaries.

We also performed immunofluorescence analysis using antibodies against YFP, which offers a more sensitive means of detecting proteins than live imaging. We thus found that YFP-ERA-1[e3'] is distributed homogenously in the zygote and becomes enriched thereafter in the AB cell (Figure 45A and B), an anterior bias that is even more pronounced in 4-cell stage embryos (Figure 46A). Intriguingly, we noted also that YFP-ERA-1[e3'] protein is slightly enriched on the plasma membrane, something that is most apparent at the boundary between anterior blastomeres such as ABa and ABp (Figure 46A).
Figure 45. YFP-ERA-1 distribution in one- and 2-cell stage embryos
A-B YFP-ERA-1[e3’] stained with Hoechst (blue), anti-GFP antibodies (green) and anti GPA-16 antibodies (red) at telophase of the first cell division (A) and in the 2-cell stage (B). The upper images show the merge, the lower ones the YFP signal alone.

To estimate the relative cell membrane enrichment of the protein in the 4-cell stage, we performed quantification using the GPA-16 staining as a cell membrane marker. This analysis revealed that YFP-ERA-1[e3’] is enriched in the plasma membranes of the anterior cells, whereas this is not the case between the posterior cells (Figure 46F). To address if the anterior enrichment of YFP-ERA-1[e3’] protein is dependent on A-P polarity, we analyzed embryos depleted of PAR-3. As anticipated from the uniform distribution of the era-1 mRNA in such embryos (see Figure 41C), we found that YFP-ERA-1[e3’] protein is now present at comparable levels in all blastomeres (Figure 46C, 46F).
Figure 46. YFP-ERA-1 distribution in 4-cell stage embryos in MEX-5/6-dependent.
A-C 4-cell stage embryos expressing YFP-ERA-1[3′e] in control (A), mex-5/6(RNAi) (B) and in par-3(RNAi) (C) conditions stained with Hoechst (blue), anti-GFP antibodies (green) and anti GPA-16 antibodies (red). The upper images show the merge, the lower ones the YFP signal alone. The signal intensities at the cell-cell boundaries of ABa/ABp and at EMS/P₂ were measured and quantified in F. Quantification of signal intensities is shown in panel F. The brightness and contrast properties of the images are equivalent resulting in seemingly overexposed YFP signal in these panels. Note that the brightness and contrast properties of the images are equivalent, resulting in seemingly overexposed YFP signal in these panels. 
D-E 4-cell stage embryos expressing YFP-ERA-1[3′p] in control (D) and in mex-5/6(RNAi) (E) conditions. The quantification of signal intensities is shown in panel F. 

Furthermore, as anticipated from the likewise distribution of the corresponding mRNA, we found that YFP[3′e] protein is present solely in anterior blastomeres, albeit at lower levels than for YFP-ERA-1[3′e] (Figure 47A), indicating that the era-1 3′UTR can drive anterior protein distribution. Moreover, we found that YFP-ERA-1[3′p] protein exhibits a homogenous distribution throughout the embryo and that it is present at higher levels than YFP-ERA-1[3′e] protein (Figure 47B). Taken together, these observations indicate that the era-1 3′UTR is needed to prevent translation of the era-1 mRNA.
**Figure 47. YFP[e3’] and YFP-ERA-1[p3’] distribution during embryogenesis**

A Snapshots of an embryo expressing YFP[e3’] imaged with dual DIC and fluorescence time-lapse microscopy. B Snapshots of an embryo expressing YFP-ERA-1[p3’] with dual DIC and fluorescence time-lapse microscopy. Note that the brightness and contrast were lowered in panel B in order to avoid blasting of the signal, indicating that the expression level of this strain is higher than two others.

**MEX-5/6 positively regulates era-1 translation via the era-1 3’UTR**

What trans-acting factor(s), if any, could be responsible for mediating the anterior translation of ERA-1 protein? The partially redundant mRNA binding zinc-finger proteins MEX-5 and MEX-6 appeared as plausible candidates since they are themselves enriched on the anterior of the zygote (Schubert et al., 2000). To address if MEX-5/6 affects the translation of ERA-1, we analyzed embryos expressing YFP-ERA-1[e3’] depleted of MEX-5/6. We found that in mex-5/6(RNAi) embryos, YFP-ERA-1[e3’] protein is greatly reduced in embryonic blastomeres (Figure 48A).
Figure 48. MEX-5/6 regulates YFP-ERA-1 translation via the era-1 3'UTR

A-C YFP signal by TimeLapse fluorescent microscope of ~30 cell stage mex-5/6(RNAi) YFP-ERA-1[e3'] (A), YFP[e3'] (B) and YFP-ERA-1[p3'] (C) embryos. Compare images to Figures 44 and 47.

This reduction at the protein level can not be explained by destabilized mRNAs, as RT-qPCR analysis revealed no difference in era-1 mRNA levels between control and mex-5/6(RNAi) embryos (Figure 49), raising the possibility of translational regulation. Similarly to YFP-ERA-1[e3'], the levels of YFP[e3'] are also diminished in mex-5/6(RNAi) (Figure 48B). These observations suggest that MEX-5/6 are needed to activate the translation of era-1 mRNA through the era-1 3’UTR. Importantly, MEX-5/6-directed translational regulation seems to involve the era-1 3’UTR, as levels of YFP-ERA-1[p3’] protein in mex-5/6(RNAi) embryos appear to be unaffected (Figure 48C).
**Figure 49.** MEX-5/6 depletion does not influence mRNA levels of ERA-1 transgenes.
Relative mRNA levels measured by RT-qPCR in YFP-ERA-1[e3'], YFP[e3'] and YFP-ERA-1[p3'] embryos with or without mex-5/6 depletion. Values are shown relative to act-1 mRNA. The experiment was performed 3 times.

Quantification of cell membrane enrichment in 4-cell stage embryos further demonstrated that interfering with MEX-5/6 leads to a drop of YFP-ERA-1[e3'], but not of YFP-ERA-1[p3'] protein levels (Figure 46B and E, quantified in 4F). Importantly, the decreased YFP-ERA-1[e3'] levels are not due to the misdistribution of era-1 mRNA in mex-5(RNAi) embryos, since the fluorescent signal in par-3(RNAi) embryos are higher than the ones observed in mex-5/6(RNAi) (Figure 46F). MEX-6 depletion alone does not alter the cell membrane signal of YFP-ERA-1[e3'] (Figure 50). Similarly, in the absence of an other anteriorly localized translational regulator MEX-3, YFP-ERA-1[e3'] levels are unaffected (Figure 50).

**Figure 50.** MEX-6 and MEX-3 depletion do not lead to decreased YFP-ERA-1[e3'] signal
Quantification of relative membrane enrichment in 4-cell stage YFP-ERA-1[e3'] embryos upon single mex-5, mex-6 and mex-3 depletion. Embryos used for quantification: n=11 (control), n=7 (mex-5(RNAi)), n=7 (mex-6(RNAi)), n=7 (mex-3(RNAi)).
Is it possible that \textit{era-1} mRNA is a direct MEX-5/6 target? MEX-5 binds to RNA motifs that contain six or more uridines within a 9-13 nucleotide window. Suggestively, we found several such motifs in the \textit{era-1} 3’UTR of \textit{C. elegans} and of the sister species, \textit{C. briggsae} (Figure 51). Of note, these AU-rich sequences are very abundant in 3’UTR’s of \textit{C. elegans} genes (Pagano et al., 2007), therefore the involvement of these motifs should be proven experimentally to establish a link between the \textit{era-1} 3’UTR and MEX-5.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure51.png}
\caption{Putative MEX-5-binding sites in the 3'UTR of \textit{C. elegans} and \textit{C. briggsae} \textit{era-1}}
\end{figure}

Putative MEX-5 binding sites (red boxes) along the \textit{era-1} 3’UTR (green) in \textit{C. briggsae} and \textit{C. elegans}. The sequence of the regions is shown below in red.

\textbf{Bioinformatic analysis of \textit{era-1}}

In the hope of gaining insight into the function of ERA-1, we sought to perform a small bioinformatic exploration, in collaboration with the Biostatistics and Bioinformatics Core Facility at EPFL.

First, we sought to identify domains and motifs in ERA-1 using the NCBI and ScanProsite webservers. We found a putative transcriptional regulator ICP4 domain in the ERA-1 sequence, but given that our YFP-fusions do not show nuclear localization, ERA-1 does not appear to be a transcriptional factor, at least not in early embryos. Of course, it is possible that endogenous ERA-1 distribution differs from that of the YFP-fusion proteins. Moreover, the ScanProsit server identified several motifs in the amino acid sequence (Figure 52A), although all are annotated as motifs with ‘high probability occurrence’, meaning that these motifs are found in numerous
amino acid sequences. Among these, the putative phosphorylation sites of protein kinase C and Casein kinase II could be of potential interest, as PKC-3 is part of the anterior PAR complex and since CSNK-1 is involved in generation of pulling forces in the zygote (Panbianco et al., 2008). Moreover, the presence of predicted myristoylation sites is indicative of the potential for autonomous membrane binding. Of note, these predicted motifs are not located in the N-terminus of ERA-1, thus their relevance is questionable.

**Figure 52. Potential motifs in ERA-1, potential related proteins identified by BLAST analysis and phylogenetic tree**

A Motifs identified with the ScanProsit software in the ERA-1 protein sequence. The four ERA-1 exons are in blue, the ruler indicates the length in amino acid. Dashed black line shows the truncated protein in the era-1(tm5854) line (see later section). B Blast performed in the NCBI website based on the ERA-1 sequence chopped into four fragments. The description of the proteins and the species where they act are indicated next to the fragment number. The score displayed by the algorithm of a given hit is indicated in blue. We excluded hypothetical proteins from the hits. C Phylogenetic tree based on ERA-1 amino acid sequence in C. elegans, C. japonica, C. brenneri, C. briggsae and C. remanei. The analysis was done with the RXML blackbox (http://embnet.vital-it.ch/raxml-bb/) based on sequence alignment performed with the MAFFT server (http://mafft.cbrc.jp/alignment/server/).

We were interested in identifying potential paralogs of ERA-1 in C. elegans and homologues in other systems. However, we failed to find paralogs in C. elegans by blasting the sequence against the genome using the BLASTn algorithm. To find potential homologues, we chopped the ERA-1 amino acid sequence into four ~100
amino acid-long fragments and performed BLASTp search (Figure 52B). We identified proteins that bear similarity scores ranging from 33 to 39 as compared to the score of ~97 for the ERA-1 sequence itself. These proteins are shown in Figure 52B and C. Out of these, given the membrane localization of ERA-1, proteins with membrane-association are potentially interesting, like a sodium-channel protein in Zebrafish, a Ton-B dependent receptor in the proteobacterium Sphingobium xenophagum or a membrane-bound lytic murein transglycosylase in the bacterium Candidatus odyssella. However, whether ERA-1 shares functional similarities with these proteins remains to be investigated.

To investigate the evolutionary conservation of ERA-1, we decided to build a phylogenetic tree derived from related nematode species based on ERA-1 protein sequence (Figure 52C). Interestingly we found that C. elegans ERA-1 has greatly diverged from the other nematodes. This suggests that ERA-1 might become redundant with other protein(s) in C. elegans during evolution. Based on this, it is possible that investigating the function of ERA-1 in related species could lead to exciting discoveries (see in the light of the next chapter).

**Attempts to analyze the phenotypic consequences of era-l depletion**

We found that RNAi-mediated depletion of era-l by feeding, soaking or injection does not lead to embryonic lethality or to any obvious defect in the adult worm. This is likely not the consequence of inefficient RNAi, as era-l mRNA is decreased to ~8% of the wild type levels following RNAi by feeding (Figure 53A). We also took advantage of a deletion allele of era-l, era-l(tm5854), that harbors a 500-bp deletion from exon 2 to exon 3 and results in a premature STOP codon in exon 3 (Figure 53B). We verified the deletion by genomic PCR and found by RT-PCR that exon 1 is expressed at a lower level in era-l(tm5854) as compared to wild type (Figure 53C and D). Based on this, we handle era-l(tm5854) as a potential hypomorphic allele, although it is most likely a null. Careful examination of these embryos and worms did not reveal any defects at any stage at any temperature.
Figure 53. RNAi-mediated depletion of era-1 and the era-1(tm5854) allele
A RT-qPCR analysis of era-1 mRNA isolated from control and era-1(RNAi) embryos. act-1 was used as control, N=2 experiments. B Molecular nature of the era-1(tm5854) allele. The 500 bp deletion between exons 2 and 3 gives rise to a premature STOP codon. C Genomic PCR confirming the 500 bp deletion using primer pairs indicated with black arrows in panel B. Here and in panel D, ‘L’ marks the DNA ladder lane; numbers indicate nucleotides sizes. D Semi-quantitative RT-PCR analysis of era-1 mRNA in wild type and era-1(tm5854) embryos using primer pairs amplifying a region in exon 1 (green arrowheads in panel B).

We also analyzed a strain carrying a 700 bp deletion starting from the 5’UTR of the era-1 gene, era-1(tm6426) (Figure 54A), which we consider a complete null. This strain is embryonic viable and displays no apparent phenotypes in adult stages. Based on this, we conclude that era-1 is not an essential gene. Interestingly, however, we found that era-1(tm6426) embryos are sensitive to partial loss of the polarity proteins, PAR-3 or PAR-2 (Figure 54B). A possible interpretation of this finding is that ERA-1 acts redundantly with partner protein(s) during development, but the exact process in which ERA-1 might function remains elusive.

Figure 54. era-1(tm6426) is sensitive to slight perturbation of polarity
A Schematics of endogenous era-1 gene and of the era-1(tm6426) allele. B Embryonic lethality following partial depletion of par-3 and par-2 in wild type and in era-1(tm6426) worms. >800 embryos were scored in total of the 8 independent par-3(RNAi) experiments and >200 embryos were scored in total of the 3 independent par-2(RNAi) experiments.
Since we thought that the lack of phenotype could result from era-1 functioning redundantly with another gene, we decided to take a candidate approach to identify such a potential partner protein. Single cell sequencing methods uncovered several mRNAs that are enriched in AB as compared to P1 (Table 1; Hashimshony et al., 2012). Besides this list, we were aware of other anteriorly-enriched mRNAs identified in the Lieb lab (Jason Lieb, personal communication). We reasoned that era-1 might function together with mRNAs that have analogous localization. Therefore, we depleted each of these genes by feeding RNAi in the wild type and the era-1(tm5854) background to reveal a potential genetic enhancement. First, we analyzed the genes that, similarly to era-1, do not cause embryonic lethality. Although we did not test the efficiency of RNAi for each gene, we did not find any trace of synthetic lethality. In the case of genes causing embryonic lethality on their own, we performed partial RNA experiments that similarly lead to no enhancement with era-1(tm5854). Our mini-screen thus failed to find conditions where the loss of era-1 leads to developmental defects.

<table>
<thead>
<tr>
<th>gene name</th>
<th>NEXTDB</th>
<th>PhenoBank</th>
<th>enhancement on era-1(tm5854)</th>
</tr>
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<tbody>
<tr>
<td>E02H4.6</td>
<td>ND</td>
<td>WT</td>
<td>no</td>
</tr>
<tr>
<td>F32D1.6</td>
<td>ND</td>
<td>WT</td>
<td>no</td>
</tr>
<tr>
<td>mig-5</td>
<td>saturated signal</td>
<td>WT</td>
<td>no</td>
</tr>
<tr>
<td>T21B10.4</td>
<td>ND</td>
<td>WT</td>
<td>no</td>
</tr>
<tr>
<td>Y59A8B.12</td>
<td>ND</td>
<td>WT</td>
<td>no</td>
</tr>
<tr>
<td>ZK1127.6</td>
<td>symmetric</td>
<td>WT</td>
<td>no</td>
</tr>
<tr>
<td>mex-3</td>
<td>asymmetric</td>
<td>100% Emb</td>
<td>no</td>
</tr>
<tr>
<td>par-3</td>
<td>ND</td>
<td>100% Emb</td>
<td>no</td>
</tr>
<tr>
<td>hmp-2</td>
<td>symmetric</td>
<td>100% Emb</td>
<td>no</td>
</tr>
<tr>
<td>Y73F8A.24</td>
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<td>70-90% Emb</td>
<td>no</td>
</tr>
<tr>
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<td>symmetric</td>
<td>Ste</td>
<td>no</td>
</tr>
<tr>
<td>erm-1</td>
<td>symmetric</td>
<td>weak Dumpy</td>
<td>ND</td>
</tr>
<tr>
<td>hsp-4</td>
<td>symmetric</td>
<td>Ste</td>
<td>ND</td>
</tr>
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</table>

Table 1. mRNAs displaying relative enrichment in AB versus P1
These genes were identified by single cell mRNA sequencing method (Hashimshony et al., 2012). The second column indicates if the transcript exhibits asymmetry in early embryos based on the in situ image in the publicly available NEXTDB. PhenoBank shows the phenotype upon depletion of the gene by RNAi, the last column display the lack of synthetic lethality/enhancement when treating era-1(tm5854) embryos with RNAi.
Phenotypic consequences of ERA-1 mislocalization

The overall conclusion from the above-mentioned experiments is that *era-1* depletion alone leads to no observable consequences. We sought to test if this was also the case in embryos in which ERA-1 is mislocalized. We did not detect embryonic lethality in the YFP-ERA-1[p3’] strain. Thus, in the presence of functional endogenous protein, the mislocalization of ERA-1 does not result in developmental problems. Note that we do not have means to test if the YFP fusion of ERA-1 is functional, as we do not have a clear phenotypic readout that we could use to address this question. It is thus possible that the apparent absence of phenotype stems from the fact that YFP-ERA-1 is not functional. We next crossed the YFP-ERA-1[p3’] strain into the *era-1(tm5854)* and into the *era-1(tm6426)* mutants to be in a condition where the sole source of ERA-1 in the embryos is the mislocalized YFP fusion. The resulting strain was embryonic viable and displayed no apparent adult phenotype. Thus, we conclude that ERA-1 mislocalization under laboratory conditions is tolerable for embryonic development of *C. elegans.*
4B. DISCUSSION

*era-1* mRNA and ERA-1 protein distribution

In this part of the thesis work, we provided evidence that the mRNA of the uncharacterized gene W02F12.3, hereby named *era-1*, is enriched in the anterior part of the *C. elegans* zygote and is inherited in anterior blastomeres. We found that analogous to the mRNA, YFP-ERA-1 protein is enriched in the anterior blastomeres where it localizes to the plasma membrane. We found that the asymmetric enrichment of *era-1* mRNA is mediated by polarity and is dependent on its 3’UTR.

Interestingly, neither *era-1*, nor another asymmetrically enriched mRNA, *mek-1*, are among the genes identified by single cell mRNA sequencing of blastomeres of being asymmetrically enriched in AB versus P₁ (Hashimshony et al., 2012). Conversely, 6 out of 13 transcripts identified in this study clearly show no asymmetric localization based on *in situ* images in the Nematode Expression Database (Table 1). This discrepancy demonstrates that the mRNA sequencing and large-scale *in situ* hybridization methods probably fail to reveal all mRNAs with asymmetric distribution. More reliable techniques and analyses, similar to those have been implemented in the case of *Drosophila* embryos (Lecuyer et al., 2007) would be needed to get clearer picture about asymmetrically enriched mRNAs in *C. elegans* embryos.

**Regulation by MEX-5**

We found that embryos depleted of MEX-5 show aberrant *era-1* mRNA localization and absence of ERA-1 protein. This translational regulation exerted by MEX-5, and potentially also its impact on mRNA localization, is mediated through the *era-1* 3’UTR. Thereby, we propose a model where MEX-5 plays a dual role in *era-1* mRNA regulation (Figure 55). First, by binding to the *era-1* 3’UTR, MEX-5 contributes to the transport of *era-1* mRNA to the anterior side of the cell during the polarization phase. Second, MEX-5 promotes translational activation of *era-1* mRNA in the AB cell in the 2-cell stage. On top of this MEX-5-mediated translational activation, it is plausible that translational repression ensures low ERA-1 levels before the 2-cell stage acting via the 3’ UTR, as our construct harboring the *pie-1* 3’UTR shows high ERA-1 protein levels already in P₀.
MEX-5 acts redundantly in several aspects with another CCCH zinc-finger protein, MEX-6 (Schubert et al., 2000). Slight polarity defects, for instance, have been observed in embryos depleted of both MEX-5 and MEX-6, while such an effect has not been reported for single depletion. It would be interesting to address if era-1 mRNA regulation is restricted to MEX-5 or if MEX-6 is likewise involved.

Is such regulation exerted by MEX-5 specific to era-1 mRNA? Although MEX-5 has been shown to regulate mRNA localization and translation, this is the first case where MEX-5 is implicated in both mRNA localization and translational activation. Thus, as opposed to the wild type situation where pos-1 mRNA is enriched in P₁ as compared to AB, in single mex-5 mutant embryos, the asymmetry at the mRNA level is no longer observed. Interestingly, however, POS-1 protein is still enriched in the posterior cell (Tenlen et al., 2006). This is no longer the case in double mex-5/6 mutant embryos, where POS-1 protein distribution is uniform (Schubert 2000), serving as another example of redundancy between MEX-5 and MEX-6. Furthermore, GLP-1 protein normally decorating the anterior plasma membranes is diminished in mex-5/6 double mutant embryos (Schubert et al., 2000), indicating a role for MEX-5/6 in translational activation of glp-1 mRNA. Of note, the mRNA

**Figure 55. Model of era-1 mRNA localization and translation regulation**

A era-1 mRNA (black curve) and MEX-5 (orange circle) are uniformly distributed in early P₀. B era-1 mRNA is asymmetrically enriched by MEX-5 mediated anterior movement through the era-1 3’UTR in late P₀. C MEX-5 activates era-1 translation, newly translated ERA-1 protein (purple diamond) is transported to the plasma membrane.
levels of *glp-1* were not investigated in this case, thus we can not rule out the possibility that the loss of GLP-1 protein enrichment is the consequence of decreased mRNA levels.

**era-1 mRNA distribution by other mechanisms**

Based on the fact that the asymmetric localization of GFP-MEX-5 in the one cell stage embryo is not as sharp as that of *era-1* mRNA (Cuenca et al., 2003) it is plausible that other factors are involved in setting up asymmetric *era-1* mRNA distribution. What could be nature of these other factors? The endosomal network, the endoplasmic reticulum and the acto-myosin network all have been implicated in contributing to mRNA localization in other instances and, suggestively, they are all enriched in the anterior side of $P_0$ (Andrews and Ahringer, 2007; Aronov et al., 2007; Balklava et al., 2007; Estrada et al., 2003; Irion and St Johnston, 2007; Munro et al., 2004; Poteryaev et al., 2005; Schmid et al., 2006; Velarde et al., 2007; Zhao et al., 2001). It is plausible that these components contribute to *era-1* mRNA localization. Testing the involvement of these components could be possible by utilizing fast-acting temperature-sensitive mutants of *nmy-2* or of the dynamin gene *dyn-1*. Moreover, during centration/rotation, when *era-1* mRNA loading in the anterior is being established, there is a relative enrichment of microtubule plus ends at the anterior side of the cell. Given this, it is plausible that *era-1* mRNA is transported with the help of kinesins, as in the case of *osk* and Vg1 mRNAs (Betley et al., 2004; Brendza et al., 2000; Yoon and Mowry, 2004). This hypothesis can be tested by RNAi-mediated depletion of the plus-end directed kinesin genes present in *C. elegans* (reviewed in Siddiqui, 2002).

**Potential function of ERA-1**

The exact function of ERA-1 remains elusive. We found that slight perturbation of polarity leads to synthetic lethality in *era-1(tm6426)* embryos. This suggests that ERA-1 functions redundantly with other protein(s) whose distribution is under the control of polarity. What could be the nature of such proteins? The Notch pathway is crucial for defining the fate of ABp by signaling stemming from $P_2$ (see Introduction on page 50). Given that YFP-ERA-1 localization exactly matches that of the Notch receptor, GLP-1, it is possible that ERA-1 influences Notch signaling.
We found that both YFP-ERA-1 fusion proteins are enriched at the cell membranes. What could be the function of ERA-1 in the cell membrane? Our BLAST analysis found several membrane-associated proteins that share similarity with ERA-1, one of them being a voltage-gated sodium channel (VGSC) protein from Zebrafish. Although VGSCs mostly function in excitable cells, there is ample evidence of their importance in nonexcitable cells regulating diverse processes, such as phagocytosis, cell motility and metastasis (reviewed in Black and Waxman, 2013), raising the possibility that VGSCs could also play a role in cells where ERA-1 is present.
5. CHARACTERIZATION OF THE EFFECT OF THE CLATHRIN HEAVY CHAIN ON FORCE GENERATION

5A. RESULTS

The clathrin heavy chain CHC-1 negatively regulates pulling forces in *C. elegans* embryos

In the course of an RNAi-based functional genomic screen (Gönczy et al., 2000), it was found that depletion of the clathrin heavy chain CHC-1 results in several phenotypes visible by DIC in one-cell embryos, which we set out to further investigate. As in other systems, clathrin plays a role in receptor-mediated endocytosis in *C. elegans* and is thus required for proper yolk intake in the oocyte (Figure 56A and B; Grant and Hirsh, 1999). We found in addition that *chc-1(RNAi)* one-cell embryos exhibit stereotyped MTOC positioning defects that are detailed below.

During centration/rotation, instead of the smooth movement of centrosomes and associated pronuclei towards the anterior that are characteristic of the wild-type, excess back and forth movements are observed in *chc-1(RNAi)* embryos (Figure 56A-D). To quantify this phenotype, we determined the velocity of the movements of centrosomes as well as their angular displacement during centration/rotation (see Figure 56E for a schematic of the quantification methods). We found that both metrics are significantly higher in *chc-1(RNAi)* embryos than in the wild type (Figure 56F and G).
Figure 56. Clathrin negatively regulates net pulling forces acting on centrosomes during centration/rotation

A, B Centrosome position in wild type (A) and chc-1(RNAi) (B) embryos monitored by time-lapse DIC microscopy. Centrosomes are marked with green and blue asterisks. Insets illustrate the depletion of yolk granules in chc-1(RNAi) embryos. Here and in other figures, time is indicated in seconds, with t=0 corresponding to pronuclear meeting unless stated otherwise, and scale bars represent 10 microns. C, D Kymographs of the areas marked by
dashed white rectangles in A and B. White line delineates the position of the nuclear envelopes of the joined pronuclei. The embryos were imaged with a frame rate of 0.5 s to acquire a kymograph with high resolution. Here and in the following cases, we used the first 150 seconds from pronuclear meeting onwards to compose the kymographs. E Schematic illustration of the quantification methods of centrosome movements during centration and of their angular displacement. F, G Average centrosome movements (F) and average angular displacement (G) in wild type and chc-1(RNAi) embryos. n=10 embryos from 3 experiments in every case. H Schematic illustrating the experiment aimed at assessing net pulling forces acting on the anterior or posterior centrosome during centration/rotation. Microtubules were severed with the laser microbeam close to the cell cortex, as indicated by the red line, and the resulting displacement of the pronuclei in the opposite direction determined. The extent of this displacement reflects the extent of net pulling forces acting on the posterior (upper panel) or anterior (lower panel) centrosome. I Average peak velocities of centrosomes following laser severing of microtubules during centration rotation. Posterior cuts (red) and anterior cuts (blue) were performed to evaluate forces acting in both directions. J Average posterior-directed peak velocities of centrosomes following laser severing of anteriorly directed astral microtubules in embryos of the indicated conditions.

As mentioned before, the net pulling force acting on spindle poles during anaphase can be estimated using laser microbeam-mediated severing of astral microtubules (Grill et al., 2001). We designed an analogous assay during centration/rotation, performing severing of astral microtubules emanating from the anterior or the posterior centrosome. The extent of anteriorly directed net pulling forces was inferred from the velocity of anteriorly directed movements following posterior severing, and that of posteriorly directed net pulling forces from the velocity of posteriorly directed movements following anterior severing (see Figure 56H for a schematic of these experiments). The resulting analysis revealed that net forces are larger on the anterior than on the posterior at this stage (Figure 56I), in line with analogous experiments in which centrosomes were targeted with a laser microbeam (Labbe et al., 2004). Moreover, we found that peak velocities following an anterior cut are lower in gpr-1/2(RNAi) embryos than in the wild-type (Figure 56J), as anticipated from the ternary complex playing a role in centration/rotation (Park and Rose, 2008). Most importantly, these experiments revealed also that net pulling forces are substantially higher both on the anterior and posterior centrosomes in chc-1(RNAi) compared to the wild-type (Figure 56I). This increase is not due merely to the depletion of yolk granules, since peak velocities after an anterior cut are indistinguishable from the wild-type in rme-2(RNAi) embryos (Figure 56J), in which yolk granules are also sparse due to defective endocytosis (Grant and Hirsh, 1999).

We also observed that even though centrosomes in chc-1(RNAi) embryos occupy a normal position by the end of centration, they are displaced prematurely towards the
posterior by the time of nuclear envelope breakdown (NEBD), suggestive of higher posterior pulling forces (Figure 57A). Nevertheless, the spindle is oriented along the AP axis in chc-1(RNAi) embryos as it is in the wild-type (Figure 57B and C).

Figure 57. Clathrin depletion leads to premature posterior spindle displacement
A Quantification of the position of centrosomes at the end of centration, nuclear envelope breakdown (NEBD) or the onset of cytokinesis in wild type (black lines) and chc-1(RNAi) embryos (grey lines). The position of the anterior (green) and the posterior (blue) MTOCs was projected onto the AP-axis of the embryo. Filled lines indicate the spindle, the dashed line the shortest path between the two centrosomes. n=10 embryos each from 3 experiments.
B, C Spindle position (B) and quantification of its alignment with the AP axis (C) at metaphase in 10 control (black) and 10 chc-1(RNAi) (grey) embryos.

An MTOC positioning phenotype was also observed thereafter in chc-1(RNAi) embryos. Thus, we found that the anterior spindle pole of chc-1(RNAi) embryos exhibits excess oscillations during anaphase (Figure 58A-D), suggestive of increased net forces pulling on the anterior spindle pole. To test whether this is the case, we performed spindle-severing experiments during anaphase and found that the average peak velocity of the liberated anterior spindle pole is higher indeed in chc-1(RNAi) embryos than in the wild-type (Figure 58E). Despite such alterations in net pulling forces, the final position of the anaphase spindle and thus the placement of the cleavage furrow are essentially unchanged in chc-1(RNAi) embryos, presumably because of the above mentioned premature posterior displacement of centrosomes at the time of NEBD (Figure 58A).

Overall, we conclude that the clathrin heavy chain negatively regulates pulling forces on the anterior side during centration/rotation and anaphase in C. elegans embryos.
Figure 58. Clathrin negatively regulates net pulling forces acting on centrosomes during anaphase

A, B Position of the anaphase spindle in wild type (A) and chc-1(RNAi) (B) embryos. Spindle poles are marked with green (anterior) and blue (posterior) asterisks.

C, D Anterior (green) and posterior (blue) spindle pole positions during anaphase in embryos shown in A and B. Spindle poles were tracked for one minute before cytokinesis onset at a frame rate of 5 s. Dashed grey oval lines represent the embryo, black asterisks mark the initial position of the spindle poles.

E Average peak velocities of anterior (green) and posterior (blue) spindle poles after spindle severing in wild type and chc-1(RNAi) embryos. n=10 embryos each from 3 experiments.
CAR-1 depletion also leads to MTOC positioning defects and results in lower CHC-1 levels

We were intrigued by the fact that embryos depleted of the cytokinesis/apoptosis/RNA-binding protein CAR-1 exhibit MTOC positioning phenotypes that appeared reminiscent of those in chc-1(RNAi) embryos (Squirrel et al., 2006). CAR-1 is a multifunctional RNA binding protein that plays a role in germline apoptosis (Boag et al., 2005), in regulating P-granule-related mRNAs (Noble et al., 2008) and in cytokinesis (Audhya et al., 2005). Intriguingly, overexpression of the CAR-1 homologue Scd6p/Lsm13p in budding yeast suppresses clathrin deficiency (Nelson and Lemmon, 1993). Although several aspects of CAR-1 function have been characterized previously in C. elegans, we focused on the fact that CAR-1 depletion leads to excess back and forth movements of centrosomes during centration/rotation, resulting in increased centrosome movements and angular displacement during centration/rotation compared to the wild type (Figure 59).
Moreover, we found that the anterior spindle pole undergoes excess oscillations during mitosis in \textit{car-1(RNAi)} embryos (Figure 60). Overall these phenotypic manifestations are similar to the ones in \textit{chc-1(RNAi)} embryos.
Therefore, we examined the distribution of clathrin in car-1(RNAi) embryos. In wild-type one-cell embryos, GFP-CHC-1 is present primarily in the cytoplasm, is enriched on the spindle and is also detectable in the vicinity of the cortex (Figure 61A). Strikingly, we found that the overall levels of GFP-CHC-1 are markedly reduced in car-1(RNAi) embryos (Figure 61B and C). How CHC-1 levels depend on CAR-1 function remains to be investigated but, regardless of the mechanism, this requirement is not bidirectional, since CAR-1 levels and distribution appear unaffected in chc-1(RNAi) embryos (Figure 61D and E). Moreover, we observed that yolk granules are not altered in car-1(RNAi) embryos (Figure 59B), further demonstrating that the chc-1(RNAi) MTOC positioning phenotype is not due to depletion of yolk granules.
Figure 61. CAR-1 depletion leads to decreased CHC-1 levels in embryos

A, B Immunofluorescence analysis of control (A) and car-1(RNAi) (B) embryos expressing GFP-CHC-1, stained with antibodies against GFP (green on the left, grey on the right); DNA is seen in blue. Insets display magnified region of the spindle (blue box) and cortex (red box). C Western blot analysis of lysates from control and car-1(RNAi) embryos expressing GFP-CHC-1, using antibodies against GFP, α-tubulin or CAR-1, as indicated. The CAR-1 antibody specifically recognizes two species (Audhya et al., 2005); asterisk denotes a non-specific band. D, E Immunofluorescence analysis of GFP-CAR-1 in control (D) and chc-1(RNAi) (E) embryos.

Panels on the left show the merge of DNA (blue), α-tubulin (red) and GFP (green), right panels show GFP alone (grey).

Overall, we conclude that CAR-1 is needed for appropriate CHC-1 levels in the early embryo. The above results indicate that the CAR-1 phenotype can be explained by the impact on CHC-1, and we thus investigated the chc-1(RNAi) phenotype in more detail to uncover how clathrin negatively regulates pulling forces.

Higher forces in chc-1(RNAi) do not arise from increased levels of cortical force generators

We set out to determine the root of the higher net pulling force phenotype upon CHC-1 depletion. We tested whether polarity is affected, but found that the distribution of P-granules is not changed in chc-1(RNAi) embryos (Figure 62A and B). Given the role of clathrin in organizing spindle pole structure in human cells (Foraker et al., 2012), we also investigated whether MTOC structure is altered in chc-1(RNAi) embryos. However,
staining with antibodies against the MTOC component TAC-1 (Bellanger and Gönczy, 2003; Le Bot et al., 2003; Srayko et al., 2003) did not reveal a difference with the wild-type during either centration/rotation or mitosis (Figure 62C-F).

Figure 62. **Polarity and centrosomes are not affected in chc-1(RNAi) embryos**

A, B Immunofluorescence analysis with PGL-1 antibodies to mark P granules in wild type (A) and chc-1(RNAi) (B) anaphase embryos. Z-projection merge images of DNA (blue), α-tubulin (green) and PGL-1 (red). C-F Immunostaining of DNA (blue), α-tubulin (green) and TAC-1 (red) in wild-type (C, D) and chc-1(RNAi) (E, F) embryos during centration/rotation and during anaphase. The insets show magnified region of TAC-1 (upper insets) and α-tubulin (lower insets) single channels.

The chc-1(RNAi) phenotype bears similarity with that of embryos depleted of Giβγ, in which cortical levels of ternary complex components and of dynein are increased because more Gα is available for interaction with GPR-1/2 (Afshar et al., 2004; Tsou et al., 2003). Therefore, we set out to test whether decreased cortical GPB-1 may be what causes the MTOC phenotype in chc-1(RNAi) embryos. We did find that cortical GPB-1 is diminished in chc-1(RNAi) embryos both during centration/rotation and mitosis (Figure 56A-B and G-H). Instead, GPB-1 accumulates in intracellular punctae that are positive for the late endosomal protein, RAB-7 (Figure 63N), indicating that clathrin is required for GPB-1 trafficking. However, unexpectedly considering the observed diminution of cortical GPB-1, we found that cortical levels of LIN-5 and of the dynein heavy chain DHC-1 are not increased in chc-1(RNAi) embryos (Figure 63C-F and I-M). This observation suggests that clathrin also promotes the presence of the ternary complex and of dynein at the cell cortex. Overall, the above analysis fails to explain why there is an increase in net pulling forces upon CHC-1 depletion.
**Table 1**

<table>
<thead>
<tr>
<th>Condition</th>
<th>LIN-5</th>
<th>DHC-1</th>
<th>LIN-5</th>
<th>DHC-1</th>
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<tr>
<td><strong>C/R</strong></td>
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<tr>
<td><strong>ANAPHASE/TELOPHASE</strong></td>
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<tr>
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<tr>
<td>chc-1(RNAi)</td>
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**Figure 1**

A-H: Representative Images of Wild Type and chc-1(RNAi) Cells at Various Stages of the Cell Cycle. 

- A: GPB-1 localization in wild type. 
- B: GPB-1 localization in chc-1(RNAi). 
- C: LIN-5 localization in wild type. 
- D: LIN-5 localization in chc-1(RNAi). 
- E: DHC-1 localization in wild type. 
- F: DHC-1 localization in chc-1(RNAi). 
- G: LIN-5 localization in wild type. 
- I: LIN-5 localization in wild type. 
- K: LIN-5 localization in wild type. 
- L: LIN-5 localization in chc-1(RNAi). 
- M: Quantification of LIN-5 and DHC-1 localization showing relative cortical enrichment. 

**Figure 2**

N: GFP-RAB-7 localization in chc-1(RNAi) cells showing increased cortical enrichment. 

**Legend**

- GPB-1: Green fluorescent protein-1
- DHC-1: Dynamic heterochromatin complex-1
- LIN-5: Lin-5 protein
- C/R: Centrosome/Rotation
- ANA/TELO: Anaphase/Telophase
- wild type: Normal cell cycle progression
- chc-1(RNAi): Cell cycle perturbation

*Note: Images and graphs are illustrative and not actual research data.*
Figure 63. The levels of cortical force generators are not increased upon *chc-1(RNAi)*, A-L Immunofluorescence analysis during centration/rotation (A-F) and anaphase/telophase (G-L) monitoring the distribution of GPB-1 (A, B and G, H), LIN-5 (C, D and I, J) or DHC-1 (E, F and K, L) in wild type and *chc-1(RNAi)* embryos, as indicated. The images on the left are merges of the above respective signals (red, also shown alone on the right) with the α-tubulin signal (green); DNA is shown in blue. Insets display magnified region of the anterior cell cortex. Note intracellular accumulation of GPB-1 upon CHC-1 depletion. M Quantification of relative cortical LIN-5 and DHC-1 enrichment during centration/rotation and anaphase/telophase in wild type (black) and *chc-1(RNAi)* embryos (grey). Intensities at the cortex and in the underlying cytoplasm were determined and expressed as a ratio. 10 embryos were analyzed for each condition. N Immunofluorescence analysis of GPB-1 localization in embryos expressing GFP-RAB-7 upon CHC-1 depletion. Lowest panel shows the merge of GFP (green) and GPB-1 (red) signals; DNA is stained in blue. Insets display magnified region of cytoplasm.

Given that depletion of CHC-1 gives rise to a particularly striking phenotype during centration/rotation, we focused further analysis on this stage and asked whether the excess back and forth movements observed in *chc-1(RNAi)* embryos requires the function of cortical force generators. To this end, we depleted simultaneously CHC-1 and individual components of the ternary complex. As shown in Figure 64, we found that impairing the function of GOA-1, GPA-16, GPR-1/2 or LIN-5 suppresses excess centrosome movements during centration/rotation in *chc-1(RNAi)* embryos. We conclude that although cortical levels of force generator components are not augmented in *chc-1(RNAi)* embryos, the increase in net pulling forces is somehow dependent on cortical force generators.
Clathrin is needed for proper organization and tension of the cortical acto-myosin network

Since clathrin is known to organize the actin network in other systems (Calabia-Linares et al., 2011; Humphries et al., 2012), we explored whether CHC-1 depletion affects the acto-myosin network in early C. elegans embryos. To this end, we performed spinning disk microscopy imaging of the cortical acto-myosin network in live embryos expressing GFP::MOE to visualize filamentous (F) actin. As reported previously (Velarde et al., 2007) and shown in Figure 65A, we observed small F-actin foci at the time of pronuclear meeting in the anterior of control embryos. Strikingly, we found that CHC-1 depletion results in larger cortical F-actin foci (Figure 65B, quantified in C). We similarly imaged embryos expressing GFP-NMY-2 to visualize non-muscle myosin. We found that instead of the large foci normally present in the anterior of control embryos (Figure 65D, Munro et al., 2004), chec-1(RNAi) embryos harbor smaller foci (Figure 65E,
quantified in F). Overall, we conclude that the acto-myosin network, whilst not absent, is perturbed upon CHC-1 depletion.

<table>
<thead>
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<th>control</th>
<th>chc-1(RNAi)</th>
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<tbody>
<tr>
<td><img src="image1" alt="GFP::MOE" /></td>
<td><img src="image2" alt="GFP::MOE" /></td>
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<tr>
<td><img src="image3" alt="GFP::NMY-2" /></td>
<td><img src="image4" alt="GFP::NMY-2" /></td>
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**Figure 65. Clathrin is needed for proper organization of the acto-myosin network in early C. elegans embryos**

A, B Cortical imaging of control (A) and chc-1(RNAi) (B) embryos expressing the actin-binding fusion protein GFP::MOE visualized with spinning disk microscopy at the onset of pronuclear migration. Insets show magnified cortical regions; some actin foci are delineated in red. C, Quantification of GFP::MOE foci size in control and chc-1(RNAi) embryos; n=8 embryos each from 3 experiments, 8-10 foci per embryo. D, E Cortical imaging of control (D) and chc-1(RNAi) (E) embryos expressing GFP-NMY-2 imaged as above. Insets show magnified region of the cortex; GFP-NMY-2 foci are delineated in red. Note that GFP-NMY-2 intensities are lower in chc-1(RNAi) embryos. F Quantification of GFP-NMY-2 foci size in control and chc-1(RNAi) embryos. Note also that apart from the difference in size, there is an apparent drop in intensity of the foci in chc-1(RNAi); n=8 embryos each from 3 experiments, 8-10 foci per embryo.

Next, we tested whether such altered organization of the cortical acto-myosin network is accompanied by diminished cortical tension. To this end, we performed cortical laser ablation (COLA) experiments at pronuclear formation (Mayer et al., 2010). In this assay, cortical tension is deduced from measuring the outward velocities of GFP-NMY-2 foci following a laser cut along the longitudinal axis of the embryo (Figure 66).
**Figure 66. Principle of Cortical Laser Ablation (COLA).** Following a longitudinal laser cut in the acto-myosin cell cortex (red line), the movement of GFP::MOE or GFP-NMY-2 foci (white structures) is monitored. Blue arrows point to one specific focus, whose motion (blue dashed rectangle) is magnified in the insets; red line marks the position of the cut. The resulting outward velocity is an indirect measure of cortical tension (Mayer et al., 2010).

As can be seen in Figure 60, these experiments demonstrated that *chc-1(RNAi)* embryos exhibit significantly lower outward velocities compared to the wild-type at pronuclear formation. We next tested whether such a decrease persists into prophase when the most dramatic phenotypic manifestations take place in *chc-1(RNAi)* embryos. Because cortical levels of NMY-2-GFP are low at this stage, we performed COLA experiment using embryos expressing GFP::MOE, which give rise to a stronger fluorescence signal at this time. Importantly, these experiments established that there is a likewise significant decrease in cortical tension during centration/rotation compared to the wild-type (Figure 68).

These experiments taken together indicate that clathrin is needed for proper organization and cortical tension of the acto-myosin network in one-cell *C. elegans* embryos.
Figure 6. Clathrin depletion leads to lower cortical tension at pronuclear formation
A, B COLA in control (A) and chc-1(RNAi) (B) embryos expressing GFP-NMY-2. Four images from a movie monitoring the cell cortex using spinning disc microscopy are shown, with t=0 corresponding to the time of cut. Blue arrows mark a specific GFP-NMY-2 focus, red line indicates the cut. The cortex is monitored until complete recovery of GFP-NMY-2 foci; the inset in the last frame shows the sealed cortex with the tracked focus coalesced. Below are the kymographs constructed from the area marked by the white rectangle, with the area contoured with the red dashed box magnified on the right to better appreciate the outwards motion (dashed red lines within the box indicate the front of the outward movement).

C Quantification of initial outward velocities from 1.5 seconds before to 5.5 seconds after the cut (time interval indicated with dashed yellow lines on the kymographs); n=8 embryos each from 3 experiments were analyzed.
Figure 68. Clathrin depletion leads to lower cortical tension at centration/rotation

A, B COLA in control (A) and chc-1(RNAi) (B) embryos expressing GFP::MOE. Four images from a movie monitoring the cell cortex using spinning disc microscopy are shown, with t=0 corresponding to the time of cut. Blue arrows mark a specific GFP::MOE focus, the red line indicates the cut. The cortex is monitored until the complete recovery of GFP::MOE, the inset in the last frame shows the sealed cortex with the tracked focus coalesced. Below are the kymographs constructed from the area marked by the white rectangle, with the area contoured with the red dashed box magnified on the right to better appreciate the outwards motion (dashed red lines within the box indicate the front of the outward movement).

C Quantification of initial outward velocities from 1.5 seconds before to 5.5 seconds after the cut (time interval indicated with dashed yellow lines on the kymographs); n=9 embryos each from 3 experiments were analyzed.
Alterations in the acto-myosin network likely cause the centrosome positioning phenotype of \textit{ chc-1(RNAi) } embryos

We reasoned that if the increase in net pulling forces upon CHC-1 depletion is a consequence of defective acto-myosin organization, then stabilizing the actin cytoskeleton should rescue the centrosome positioning phenotype of \textit{ chc-1(RNAi) } embryos. Jasplakinolide is a macrocyclic peptide that promotes F-actin formation by stimulating filament nucleation (Bubb et al., 1994). We subjected embryos to Jasplakinolide during centration/rotation by piercing holes on the eggshell using the laser microbeam, thus ensuring temporal control to drug exposure and avoiding potential complications due to earlier requirements of the acto-myosin network in A-P polarity (Hill and Strome, 1988). Strikingly, we found that whereas the drug does not alter centrosome movements in the wild-type (Figure 69A-F, quantified in I), Jasplakinolide alleviates excess movements of \textit{ chc-1(RNAi) } embryos (Figure 69 C-H, quantified in I). We observed an analogous rescue during anaphase (Figure 69J and K). We conclude that alterations in the acto-myosin network are likely responsible for the MTOC positioning phenotype of embryos depleted of clathrin.
Figure 69. Stabilizing the acto-myosin network alleviates the *chc-1(RNAi)* phenotypes at centration/rotation and at anaphase

A-D Centration/rotation in control (A, B) and *chc-1(RNAi)* (C, D) embryos treated with 0.5% DMSO (A, C) or 70µM Jasplakinolide in 0.5% DMSO (B, D) and monitored by time-lapse
microscopy. Centrosomes are marked with white asterisks. Note that the image quality is less good on this microscope equipped with the laser microbeam needed to pierce the eggshell; the same holds for the next two figures. E-H Kymographs corresponding to the white rectangle depicted in A-D. I Angular displacement of centrosomes in all experimental conditions; n=10 embryos each from 3 experiments in each case. Note that treating chc-1(RNAi) embryos with DMSO alone leads to a slight drop in the angular displacement compared to untreated chc-1(RNAi) embryos (see Figure 49). Note also that Jasplakinolide led to defective cytokinesis of chc-1(RNAi), but not control, embryos (data not shown), suggesting that CHC-1 depletion sensitizes embryos to acto-myosin perturbations. J-K Anaphase spindle pole oscillations following Jasplakinolide treatment in wild type (J) and in chc-1(RNAi) (K) embryos. n=6; representative embryos are shown.

The above findings raise the possibility that small alterations in the acto-myosin network in otherwise wild-type embryos might result in centrosome positioning phenotypes reminiscent of those observed in chc-1(RNAi) embryos. We tested this prediction in two ways. First, we subjected embryos to increasing concentrations of the F-actin destabilizing drug Latrunculin A (LatA) during centration/rotation. Whereas treating embryos with high concentration of LatA (240µM) completely depolymerizes actin as judged by GFP::MOE cortical imaging (compare Figure 70A with C) lower levels of the drug (20µM) lead to an accumulation of GFP::MOE foci analogous to that observed in chc-1(RNAi) embryos (Figure 70B, quantified in D).

Figure 70. Slight destabilization of the actin network results in the formation of GFP::MOE foci analogous to that observed in chc-1(RNAi)
A-C Cortical snapshot of embryos expressing GFP::MOE at centration/rotation treated with 0.5% DMSO (A), 20µM (B) and 240µM (C) Latrunculin A. Quantification is shown in D. 10 embryos each were quantified from 2 independent experiments.

Moreover, we found that embryos exposed to low (10 µM) or high (240 µM) concentrations of LatA are indistinguishable from control embryos with respect to centrosome positioning (Figure 71A and C). In sharp contrast, embryos subjected to intermediate concentrations (20 µM and 50 µM) of the drug exhibit excess back and
forth movements of centrosomes and excess angular displacement (Figure 71 B, quantified in G).

Figure 71. Partial disruption of the actin network resembles the chc-1(RNAi) phenotype

A-C Centration/rotation in embryos treated with 0 (A), 20 (B) or 240 (C) µM latrunculin A and monitored by time-lapse microscopy. Centrosomes are marked with white asterisks.

D-F Kymographs corresponding to the white rectangle depicted in A-C. G Angular displacement of centrosomes in all experimental conditions; n=10 embryos each from 3 experiments were analyzed. The corresponding DMSO controls did not affect angular displacement.

As a second means to impart small alterations to the acto-myosin network, we utilized embryos homozygous for the fast inactivating temperature-sensitive allele nmy-2(ne3409), which is indistinguishable from the wild-type at the permissive temperature of 16°C and from nmy-2(RNAi) at the restrictive temperature of 25°C (Liu et al., 2010). We reasoned that we might observe a centrosome positioning phenotype at intermediate temperatures. As shown in Figure 72B we indeed found excess centrosome movements during centration/rotation at 20 °C, with a corresponding increased angular displacement (Figure 74G). Overall, we conclude that slight impairment of the acto-myosin network leads to a centrosome positioning phenotype analogous to that observed after depleting clathrin.
Figure 72. Partial disruption of myosin function resembles the che-1(RNAi) phenotype
A-F Centration/rotation in nmy-2(ne3409) temperature sensitive embryos imaged with time-lapse DIC microscopy at 18°C (A), 20°C (B) or 24°C (C). D-F Kymographs corresponding to the white rectangle depicted in A-C. G Angular displacement of centrosomes in all experimental conditions; n=10 embryos each from 3 experiments were analyzed. Note that embryos imaged at 20 and 21°C, as well as 24 and 25°C, were pooled for the analysis. Wild type embryos at 25°C do not differ from ones at lower temperature in terms of angular displacement (not shown).
5B. DISCUSSION

In this study, we uncovered that clathrin negatively regulates pulling forces acting on centrosomes and spindle poles in one-cell *C. elegans* embryos. Furthermore, we demonstrated that clathrin maintains proper tension of the acto-myosin cortex, which likely explains how clathrin participates in the control of centrosome positioning.

Clathrin, as a regulator of the acto-myosin network

There are other conditions besides *chc-1*(RNAi) in which centrosomes undergo excess back and forth movements during centration/rotation, as well as excess spindle oscillations during anaphase, including upon depletion of GPB-1 or CSNK-1 (Tsou et al., 2003; Afshar et al., 2004; Panbianco et al., 2008). In both cases, the increase in net pulling forces is accompanied by elevated levels of cortical force generators. By contrast, we found that this is not the case in *chc-1*(RNAi) embryos and thus inferred that clathrin must modulate force generation by another means. We discovered that this involves the acto-myosin network. We found that the organization and tension of the actin-myosin cortex are impaired upon clathrin depletion in *C. elegans* embryos. This appears to be causative of the *chc-1*(RNAi) MTOC positioning phenotype since stabilizing the actin cytoskeleton alleviates these phenotypic manifestations. The contribution of clathrin for an intact acto-myosin network has been reported in other systems. Thus, clathrin located on endosomes plays a role in organizing the actin network at the immunological synapase in T-cells (Calabia-Linares et al., 2011). Moreover, clathrin is needed for actin polymerization promoted by vaccinia viral infection (Humphries et al., 2012). Our findings contribute to this body of work and underscore the influence of clathrin on acto-myosin function in a range of biological settings. The interaction between clathrin and actin in other systems occurs via the clathrin light chain and the huntingtin interacting protein 1 related (*hipr-1* in *C. elegans*) (Boettner et al., 2011; Poupon et al., 2008; Wilbur et al., 2008). However, we found that neither the depletion of the clathrin light chain *culc-1* nor of *hipr-1* leads to MTOC positioning defects in *C. elegans* (data not shown). This raises the possibility that there may be a novel mechanism through which the clathrin heavy chain interacts with actin in the worm.
Is clathrin-dependent modulation of the acto-myosin network distinct from its well-known role in endocytosis? A definite answer to this important question is difficult to obtain. First, endocytosis in general affects the actin cytoskeleton and reciprocally, making it challenging to analyze the two processes separately (reviewed in Galletta and Cooper, 2009). Second, inhibiting endocytosis in one-cell *C. elegans* embryos using a dynamin mutant results in lower levels of cortical GPB-1, thus likely influencing force generation via increased cortical dynein (Thyagarajan et al., 2011). This could explain the clathrin-like centration/rotation phenotype observed upon depletion of the early endosomal protein RAB-5 (Hyenne et al., 2012). Note also that RAB-5 localization is slightly affected in * chc-1*(RNAi) embryos (Audhya et al., 2007), but whether CHC-1 may be altered upon RAB-5 depletion is not known. Despite these limitations, our results lead us to favor the view that the impact of clathrin on MTOC is uncoupled from its role in endocytosis. Indeed, we found that *rme-2*(RNAi) embryos, in which endocytosis is altered (Grant and Hirsh, 1999), do not exhibit an MTOC positioning phenotype. Conversely, *car-1*(RNAi) embryos, in which endocytosis is not altered, but in which CHC-1 levels are compromised, exhibit an MTOC positioning phenotype analogous to that of * chc-1*(RNAi) embryos.

**Slight perturbation in the actin network and force generation**

An involvement of the acto-myosin network during centration/rotation has been proposed previously (Goulding et al., 2007). Based on the movements of cortical foci of NMY-2-GFP, it was hypothesized that acto-myosin-derived forces can be directed towards either the anterior or the posterior of the embryo, and that LET-99 favors anterior-directed movements and thus centration (Goulding et al., 2007). Although there might be a slight mislocalization of LET-99 in * chc-1*(RNAi) embryos, perhaps as a result of perturbations in the acto-myosin cytoskeleton (data not shown), the centration/rotation phenotype of * chc-1*(RNAi) embryos is not suppressed by removing LET-99 (data not shown), indicating that LET-99 mislocalization cannot be causative in this case.

How, then, can perturbations in the acto-myosin network lead to the generation of higher net pulling forces acting on centrosomes and spindle poles of one-cell *C. elegans* embryos? We found that the angular displacement of centrosomes
during centration/rotation differs from the wild type only when the acto-myosin network is disturbed slightly, but not when it is compromised more severely. These observations are consistent with predictions from 3D simulations of anaphase spindle positioning, whereby medium levels of cortical rigidity were predicted to result in increased oscillations compared to either lower or higher cortical rigidity (Kozlowski et al., 2007). Inspired by this work, in collaboration with a physicist in the lab, Alessandro de Simone, we set out to model centrosome movements during prophase as a function of cortical rigidity. We were able to simulate the experimental data, whereby medium cortical rigidity results in maximal centrosome oscillations. The rationale behind such a force curve is that a cortex with medium rigidity, as in chc-1(RNAi) embryos, can stretch more upon being pulled whilst maintaining the interaction between the force generator and the depolymerizing microtubule, leading to higher total work (Figure 73). We note that large membrane invaginations are indeed observed in embryos that have likely impaired cortical rigidity as a result from partial depletion of NMY-2, although overall spindle positioning appears unaffected in such embryos as in chc-1(RNAi) embryos (Redemann et al., 2010).

**Figure 73. Model describing the relationship between force generation and cortical tension**

Model of cortical force generation. The depolymerizing microtubule bound to a cortical force generator stretches the cortex and thus experiences a pulling reaction force. For high cortical rigidity, a strong elastic force is applied, but the force generator detaches rapidly. For medium cortical rigidity, the stretch reaches the maximum elongation before the force generator detaches, resulting in high total forces (i.e. work). For low cortical rigidity, the stretch also reaches the maximum elongation, but the cortex is too loose to apply a large force.
Does the model we propose here for centration/rotation apply during anaphase? Intriguingly, treating mitotic embryos with the actin-depolymerizing agent Latrunculin A leads to an increase of net forces pulling on the anterior spindle pole (Afshar et al., 2010; Berends et al., 2013). However, this is the case either with high (Afshar et al., 2010; Berends et al., 2013) or intermediate (data not shown) levels of Latrunculin A. Although it may be that the effective drug concentration inside the embryo is lower during mitosis, thus resulting in medium cortical rigidity even with high levels of the drug, more work will be needed to elucidate whether the mechanisms at play during centration/rotation apply during anaphase.

A requirement for balanced levels of cortical tension may extend to human cells, in which perturbation of the acto-myosin network also affects spindle positioning on a uniform fibronectin substrate (Toyoshima and Nishida, 2007). It would be interesting to test whether the depletion of clathrin likewise affects spindle positioning in that system. We note that the cortical acto-myosin network also plays a critical role during asymmetric division of Q neuroblast in *C. elegans*, where asymmetric contractile forces are key for generating daughter cells of different sizes (Ou et al., 2010). Our findings indicate that modulation of cortical tension can be harnessed to ensure proper centrosome positioning in different biological systems.

The clathrin project was conducted as a collaboration between several people in the lab. The anterior cytoplasmic cuts (Figure 56J), the anaphase spindle severing experiments (Figure 58E), the GPB-1 immunostaining (Figure 73A, B, G and H) and the experiments testing the dependency of the * chc-1(RNAi)* phenotype on the force generator complex (Figure 64) were performed by Kalyani Thyagarajan a previous PhD student in the lab, the modeling part (Figure 66) was done by Alessandro de Simone and Sylvain Träger contributed in the CAR-1 part.

This work has been published in *Development*:

6. MATERIALS AND METHODS

Worm strains and RNAi

Wild type N2 (C. elegans), wild type AF16 (C. briggsae), lin-5(ev571he63) (Fisk Green et al., 2004), par-3(it71) (Kemphues et al., 1988), era-1(tm5854) and era-1(tm6426) strains were maintained at 20-24°C. Transgenic worms expressing GFP-LIN-5 (gift from Myeko D. Mana and Fabio Piano), lin-5 mRNA with recoded nucleotides 1-376 (Galli et al., 2011), YFP-GPR-1 (Redemann et al., 2011), YFP-ERA-1-era-1 3’ UTR, YFP-ERA-1-pie-1 3’UTR, GFP-RAB-7 (gift from Barth Grant), GFP-NMY-2 (Munro et al., 2004), GFP::MOE (Velarde et al., 2007), GFP-CHC-1 (Greener et al., 2001) were maintained at 24°C. lin-5(ev571) (Lorson et al., 2000) was kept at 16°C and shifted to 24°C for 24 hours before the experiments unless otherwise stated. The nmy-2(ne3409) temperature sensitive strain (Liu et al., 2010) was kept at 16°C, dissected in M9 stored at 16°C and shifted to the indicated temperatures at the time of pronuclear meeting. Transgenic lines were generated by ballistic bombardment in the unc-119(ed3) strain (Praitis et al., 2001).

Bacterial feeding strains for spd-5, aspm-1, zyg-12, tbb-1, mex-5, rme-2, car-1, par-3, era-1 were from the Vidal library (Rual et al., 2004), that for par-2 from the Ahringer library (Fraser et al., 2000). Clones for dhc-1(RNAi) (Cockell et al., 2004), gpa-16, goa-1 and gpr-1/2(RNAi) (Colombo et al., 2003) were described before. The RNAi feeding strain targeting the first 376 nucleotides of lin-5 was a gift from Sander van den Heuvel. RNAi was performed by feeding L3-L4 animals at 24°C for 20-24 hours for all the genes except for gpr-1/2 where the incubation was performed at 20°C for 48 hours.

Recoding of the entire lin-5 coding region was performed by manually changing each codon, considering the codon usage properties of C. elegans. Of note, in some cases, the most optimal codon had to be downgraded in order to introduce the most changes possible. As a result, the codon adaptation index of lin-5[rec] was comparable to that of lin-5.
**Microscopy, spindle severing, drug treatment and image analysis**

Embryos were observed by time-lapse DIC microscopy as described (Gönczy et al., 1999). Spindle pole oscillation was tracked by a MatLab code written by Aitana Neves De Silva. Centrosome movements during centration were determined by automatically tracking the position of the associated pronuclei using a MatLab script computing the average absolute movement of centrosomes every 5 seconds for 10 frames following pronuclear meeting. The average angular displacement during centration/rotation was calculated using ImageJ, with the X and Y coordinates of the two centrosomes computed every 5 seconds for 10 frames after pronuclear meeting. The temperature control device used for imaging *nmy-2(ne3409)* embryos was described previously (Nguyen-Ngoc et al., 2007).

Spindle severing experiments were performed essentially as described (Afshar et al., 2004) using a Leica LMD microscope equipped with a pulsed N2 laser (λ=337nm). Astral microtubules were severed during centration/rotation either anterior or posterior from the centrosome/pronuclear complex, whereas the spindle was severed at the onset of anaphase. Tracking of the MTOCs and calculation of peak velocities was performed as described (Grill et al., 2001) using the MatLab code mentioned above.

For drug treatment, the Leica LMD microscope was used to pierce a hole in the eggshell of pseudocleavage stage embryos bathed in Jasplakinolide (Lifetechnologies) or Latrunculin A (MerckMillipore), both resuspended in 0.5% DMSO. Embryos were then imaged every 5 seconds until the end of the first cell cycle.

Imaging of NMY-2-GFP and GFP::MOE was performed using an inverted Zeiss Axiovert 200 microscope with a 60x 1.4 NA lens, acquiring cortical images with a COOLSNAP HQ B/W camera every 500 ms using 250 ms excitation with a 488nm Solid State Laser. Transmission light was used every minute to unambiguously identify the embryo stage. We used ImageJ to manually mark cortical foci and determine their size, applying an empirical threshold to distinguish between two foci; 8-10 foci were analyzed per embryo.

For Cortical Laser Ablation experiments (COLA) (Mayer et al., 2010), we used an inverted Zeiss Axiovert 200 microscope equipped with a 355nm pulsed UV laser for ablation. Images were acquired with a frame rate of 500 ms using 250 ms excitation time with a 488nm Argon laser. The ~10 µm ablation was performed on the
anterior cortex along the longitudinal axis either at pronuclear formation (GFP-NMY-2 embryos) or at the onset of centration/rotation (GFP::MOE embryos). Only those embryos in which the cut sealed and that divided normally were analyzed. We used ImageJ to manually track the foci over 15 frames (3 prior to the cut and 11 afterwards) and calculate their velocity (tracking 5-10 foci or 2-3 foci per embryo for GFP-NMY-2 and GFP::MOE, respectively). The mean velocity per embryo was averaged and plotted over time. Statistical analysis was performed on the velocities measured 500 ms after the cut. The values reported here for control embryos during pronuclear migration are larger than in the literature (Mayer et al., 2010), most likely because in the previous work imaging began only 5 seconds after the cut, as compared to 500 ms here.

Dual time-lapse DIC and fluorescence imaging was performed on a Zeiss Axioplan 2 as described (Bellanger and Gonczy 2003). The motorized filter wheel, two external shutters, and the 1,392 x 1,040 pixels 12-bit Photometrics CoolSNAP ES2 were controlled by µManager. Images were acquired with an exposure time of 10-100ms for the DIC and 300 ms for the fluorescence channel using the Zeiss Filter Set 10 (GFP). The embryos were allowed to develop under the coverslip without imaging and snapshots taken at the indicating times.

**Digoxigenin in situ hybridization and single molecular FISH**

Digoxigenin-labeled single stranded DNA probes were prepared using the PCR DIG Probe Synthesis Kit (Roche) following the manufacturer’s instructions. Briefly, the plasmid carrying the gene of interest served as template in a PCR-based reaction and a reverse primer binding to the 3’UTR was added together with the DIG-11-dUTP containing dNTP mix. The resulting PCR product was ethanol-precipitated and resuspended in hybridization buffer (50% deionized formamide, 5x SSC, 100 µg/ml sonicated salmon testis DNA, 100 µg/ml yeast tRNA, 100 µg/ml heparin, 0.1% Tween-20) at a concentration of 50 ng/µl, boiled at 95°C for 75 minutes to reduce its size (Seydoux and Fire, 1995) and stored at -20°C.

The in situ hybridization protocol we developed is a combination of two previously available procedures (Motohashi et al., 2006; Seydoux and Fire, 1995).
Gravid hermaphrodites were bleached and the resulting embryos placed on a poly-L-lysine coated slide in a total volume of 7µl. Then a coverslip was gently deposited on the embryos, and the freeze-crack method was used to remove their eggshell. The slides were then submerged into 150 ml -20°C methanol and thereafter into sequential mixtures of methanol and Hepes-PBS-formaldehyde solutions at 4°C (1x PBS, 75mM Hepes pH=6.9, 0.03% EGTA, 1.5 mM MgSO₄, 1.6% formaldehyde; ratios of water:methanol:Hepes-PBS-formaldehyde being first 50:15:35, then 50:25:25 and finally 50:35:15), for 2 minutes each, before fixation in 3.6% formaldehyde in Hepes-PBS-formaldehyde for 20 minutes at 4°C. Proteinase K (5.6 µg/ml) digestion was then performed for 10 minutes at 24°C and then stopped by submerging the slides into 2 g/l glycine dissolved in PBS for 2 min followed by 2 washes in PBS-Tween (0.1%) (PBS-T) for 2 minutes each. The slides were then re-fixed in Hepes-PBS-formaldehyde for 50 minutes at room temperature, washed twice with PBS-T for 5 minutes each, treated with 2 mg/ml glycine for 5 minutes and washed again once with PBS-T. Slides were then submerged into Formamide-SSC solution (50% formamide-5xSSC-100 µg/ml heparin, 0.1% Tween-20), mixed with PBS-T in a 1:1 ratio for 10 minutes and thereafter in Formamide-SSC for an other 10 minutes. Pre-hybridization was carried out in 50 µl hybridization buffer (50% deionized formamide 50%, 5xSSC (pH=7), 100 µg/ml sonicated salmon testis DNA, 100 µg/ml yeast tRNA, 100 µg/ml heparin 0.1% Tween 20) for 1 hour at 48°C, after which the probe was applied at a concentration of 40 ng/µl in 50 µl overnight, again at 48°C. To prevent evaporation, parafilm squares glued with rubber cement were mounted on the slides. The probe was washed off twice with Formamide-SSC-PBS-T (1:1) for 10 minutes and then 4 times using 0.1% Chaps in 0.8x PBS at 48°C for 20 minutes each, after which a blocking step was included using 0.1%BSA - 0.1%Triton-X PBS for 1.5 hours at room temperature. The alkaline-phosphatase (AP)-conjugated anti-DIG antibody (Roche) was diluted in 1:2000 in 1x PBS and incubated at 4°C overnight. The slides were washed 5 times with 0.1%BSA - 0.1%Triton-X in PBS and twice with staining buffer (100 mM Tris-HCl pH=9.5), 100mM NaCl, 5mM MgCl₂, 1mM Levamisol, 0.1% Tween-20); the signal was developed using a mixture of 333 µg/ml NBT (Roche) and 70 µg/ml BCIP (AppliChem) for 20-60 minutes at room temperature. The slides were then washed, stained with 1mg/ml Hoechst 33258 (Sigma), mounted and stored at 4°C. All in situ hybridization experiments were performed at least twice.
and representative embryos are shown in the figure panels. Moreover, control embryos were included on one side of the slide in each instance.

The fluorescent probes for single molecule FISH were designed and synthesized by Biosearch Technologies. The technique was executed mostly as advised on the vendor’s website (http://www.biosearchtech.com/assets/bti_custom_stellaris_celegans_protocol.pdf) with one exception: embryos were treated as for the conventional in situ method (i.e. using bleaching and freeze-cracking). The diluted probes (40 nM for lin-5 and 250 nM for gfp) were incubated overnight (at 30°C for lin-5 and at 37°C for gfp). 1mg/ml Hoechst 33258 (Sigma) was used to counterstain the DNA. To visualize single molecules, we used the wide-field setup of a Zeiss LSM700 microscope. To quantify the enrichment around centrosomes, optical slices in Z were captured with the LSM700 confocal microscope and Z-projections performed. Thereafter, using ImageJ, a circle within the centrosome was drawn and the average pixel intensity measured in that region of interest. The average pixel intensities were also measured in the cytoplasm and the two values expressed as a ratio. The background intensity was negligible.

Quantification of relative era-1 mRNA enrichment was performed by measuring 1.) mean intensities in the cytoplasm of AB and P1, 2.) of the background, and 3.) in later stage embryos where the hybridization did not give any signal, but considerable amount of intensity arises from the yolk granules (‘empty’ embryos). Then the multiplicative inverse of the values was taken and after subtraction of the background, the values were expressed as ratio to the intensities of the ‘empty’ embryos.

RT-qPCR

To measure mRNA levels by qRT-PCR, embryos were bleached and total mRNA isolated using the trizol-chloroform extraction method (Portman, 2006). 250 µg of total RNA was then reverse transcribed using poly-dT primers. Specific primer pairs were used to amplify the cDNA with the Power SYBR Green PCR mix (Applied Biosystems). The PCR reaction was performed in the 7900HT Fast Real-Time PCR System machine (Applied Biosystems) with the SDS2.4 software using 60°C for
annealing and 72°C for elongation. The ΔΔCT method was used to quantify mRNA levels relative to the reference gene, act-1. The primer sequences were as follow:

*act-1* F - AGTCCGCGGAATCCACGAG
*act-1* R - CTTGATCTTCATGGTGATG
*lin-5* F - TTCCCTGCGATAATGGACC
*lin-5* R - TTCTCGCGATAATGGACC
*lin-5[rec] F* - CCGGAACGACATCTGTAGCC
*lin-5[rec] R* - AATGGCTGTTTAAATTCGCC
*yfp F* - GGGAACTACAAGACGTGC
*yfp R* - GTGTCCAAGAATGTTTCC

**Antibodies, western blotting and indirect immunofluorescence**

SDS-PAGE and western blot analyses were performed according to standard protocols. Antibodies against GFP (Roche, mouse, 1/500), CAR-1 (gift from Karen Oegama and Jon Audhya ( Audhya et al., 2005 ) rabbit, 1/1000), LIN-5 (1/1000, Nguyen-Ngoc, 2008) and α-tubulin (DM1A, Sigma, mouse, 1/2000) were used as primary antibodies. HRP-conjugated goat anti-rabbit and anti-mouse secondary antibodies (Promega) were used at 1/5000 and the signal detected using standard chemiluminescence (Roche).

For immunostaining, embryos were fixed in methanol at -20°C for one hour (except for 15 minutes in the case of GPR-1/2) followed by overnight incubation at 4°C with primary antibodies. Primary mouse antibodies against α-tubulin (1/300; DM1A, Sigma), GFP (1/100; MAB3580, Millipore) were used together with rabbit antibodies against GPA-16 (1/200, Afhsar et al., 2004), GPB-1 (1/200; Thyagarajan et al., 2011), GPR-1/2 (1/100, Afshar et al., 2010) LIN-5 (1/300; Nguyen-Ngoc et al., 2007), DHC-1 (1/100; Kotak et al., 2012), PGL-1 (1/300, Kawasaki et al., 1998) or TAC-1 (1/500, Bellanger and Gonczy, 2003). Secondary antibodies were Alexa488-conjugated goat anti-mouse (1/500; Molecular Probes) and Cy3-conjugated goat anti-rabbit (1/1000; Dianova). Slides were counterstained with 1mg/ml Hoechst 33258 (Sigma) to visualize DNA. Images were acquired on an LSM700 confocal microscope (Zeiss) and processed in ImageJ and Adobe Photoshop, maintaining relative image intensities within a series.
Fluorescence Recovery After Complete Photobleaching (FRAcP) and Fluorescence Loss in Photobleaching (FLIP)

Embryos expressing GFP-LIN-5 were analyzed using a Zeiss LSM700 confocal microscope. For FRAcP, we started photobleaching at the time of pronuclear migration, using the 40X objective and activating the 488 nm laser at 100% laser power with an open pinhole. Bleaching was carried out for 60 sec in the middle plane of the embryo, followed by 45 seconds each in the upper and lower halves of the embryo. This resulted in embryos at the stage of nuclear envelope breakdown with a ~95% reduction in fluorescence throughout the whole cell. After 1 minute of recovery without recording, the embryos were imaged with the 40X objective every 3 seconds with an open pinhole until mitosis was completed. Fluorescence intensities were determined using ImageJ by measuring average intensities in every frame (3 seconds) by drawing a circle around the anterior centrosome and a polygon in the anterior cytoplasm, between the cortex and the black region just outside the centrosome.

FLIP experiments were performed with the same microscope using the same laser settings. Photobleaching was conducted in a 7x7 \( \mu \text{m}^2 \) region of the anterior cytoplasm for one minute starting at the onset of posterior spindle displacement. The anterior cytoplasm and centrosome were analyzed in this experiment to avoid the posterior centrosome reaching the bleached region during anaphase spindle elongation. Fluorescence intensities of the cytoplasm and of the centrosome were measured before and after the bleach (i.e. at metaphase and late anaphase/telophase) and expressed as a ratio. Unbleached embryos served as control.

Morpholino treatment

The following morpholino oligonucleotides (MO, Gene Tools) were used:
Control MO - CCTCTTACCTCAGTTACAATTTATA
\( lin-5 \) MO – ACAACTGATGTGCTCACGCTCATTC

The morpholinos were dissolved in water and diluted in M9 just prior to the experiment at a working concentration of 100\( \mu \)M. Gonad injection was performed with a Zeiss Axiovert S100 microscope equipped with an Eppendorf Transjector 5246.
and the embryos analyzed after an incubation time of 7-9 hours. We found a clear meiotic phenotype in 13/24 early embryos (1-8 cell stage) upon injection of freshly dissolved MOs from two different batches obtained from the vendor. We speculate that the reason for such incomplete penetrance may be due to the injection not always being successful and/or the incubation period after injection being insufficient. We noted also that the efficiency of the MOs decreased after the stock solution was stored at room temperature for 45 days, with only 1/10 embryos exhibiting meiotic defects following injection in that case. The results of these experiments are summarized in Table 1.

<table>
<thead>
<tr>
<th>Morpholino stock</th>
<th>A</th>
<th>B</th>
<th>B'</th>
<th>C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Strong meiotic defect after injection</td>
<td>ND</td>
<td>5(10)</td>
<td>1 (10)</td>
<td>8(14)</td>
</tr>
<tr>
<td>Decreased posterior spindle pole oscillation in P₀ after eggshell piercing</td>
<td>5(5)</td>
<td>ND</td>
<td>3(15)</td>
<td>ND</td>
</tr>
<tr>
<td>Decreased posterior pulling forces in P₀ after eggshell piercing</td>
<td>yes (10)</td>
<td>ND</td>
<td>ND</td>
<td>yes (5)</td>
</tr>
</tbody>
</table>

Table 1. Efficiency of different MO stocks tested by injection and eggshell piercing. A, B and C indicate three separately synthesized MO stocks. The experiments in the case of stocks A, B and C were performed within two weeks of dissolving the MO, B’ is stock B stored for 45 days at room temperature.

To subject one-cell embryos to MOs, the MOs were mixed with 2% P₀ Lipofectamine 2000 (LF, Life Technologies) and the reaction mix incubated at room temperature for 20 minutes to allow liposomes to form. The embryos were placed in M9 containing the LF-MO mix and the eggshell was pierced at pseudocleavage with the pulsed laser of the LMD microscope.

Statistical analysis

All statistical analysis was performed using unpaired Student’s t-test in Excel. An F-test was run on the samples beforehand to decide if Type2 (in case F test < 0.05) or Type3 (in case F test > 0.05) unpaired t-test should be applied. *: p<0.05, **: p<0.01.
The error bars in the graphs show the standard error of the mean.
7. ACKNOWLEDGMENTS

First of all, I would like to express how thankful I am to my supervisor, Pierre. He has been giving me huge amount of support during these years, both professionally and personally. I am truly happy that I got the opportunity to work with him and to learn from him. He doubted when I was too positive and encouraged when I become too negative, thereby creating permanent motivation in me. He is an outstanding group leader and a very reasonable boss (how come he was fine with my obsessed globetrotter phenotype during these 4 years!?). My future bosses will be in a hard situation: the threshold Pierre set up is very high. Köszönöm, Pierre!

Pierre’s spirit spreads in his lab and gets incorporated in the lab members. The Gönczy lab is an excellent place to work in, where people are helpful, they think with you, they give you advice and they really care about you. This is very apparent during the super interactive lab meetings – one receives great ideas for half a year that are, if things go well, are tested for the next lab meeting. Here I am thanking all of them with whom I had the chance to work. Aitana, thanks for the MatLab codes and for the great concert in Cully! Alessandro, great collaboration in the clathrin project, and tons of fun CPs, obviously. Alex, you always get the point and have awesome ideas and thanks for the comments on my carving skiing style! Benita, I will never forget: ‘everyone is crazy in a way in this lab’, it was very reassuring! Christian, fricoius-amico-amore, you would deserve a whole chapter here; it’s really nice I can be among those 10000 people you know! Coralie, I don’t know if I am now more grateful for the injections or for the delicious cakes you prepared; but it doesn’t matter, thanks a lot for both! Daiju, I really liked your biochemical ideas, it’s a pity I couldn’t explore them all! Debora, your Nespresso capsules saved me very often…! Fernando, you were right – at some point one gets calmed down; but I did what you asked me to do in the Satellite 4 years ago! Isabelle, we had nice time in the wineyards in Ollon, now I regret I didn’t join you guys more often! Kalyani, your never-fading smile and joyful mood gave me lots of energy! And obviously, thanks for the collaboration in the clathrin story! Katy, you gave me tons of nice ideas and thanks for showing me the non-obvious parts of the LMD system. Lukas, good luck with your money making, and don’t forget to call me if you are looking for someone to give a share! Melina, I was happy to work with you and to show you some techniques – good luck.
with your PhD! Meritxell, I really enjoyed the Xmas party in 2012 with the hot ABBA girl, and the ‘how to put on a baby sling’ session. Nicola, your minivan was so great that brought us to Verbier, I hope to do some stuff together again! Paul, good luck for the new adventures (I am not talking about science now…). Sachin, thanks for spotting the lin-5 mRNA localization and for being hungry any time for scientific discussions. I learned a lot from you! Simon, even though I had to do the laundry very often, we had some great parties together. All the best with the kiwis! Veronika, I am amazed that you can bike so much after work... Slavic spirit, no question! Virginie, thanks for being a great neighbour and for the useful comments on the papers. Zhou, thanks for all the weird chinese sweets and I am hoping that you will find fun in your new life. Yemima, many thanks for opening the doors for me leading to the exciting realm of C. elegans embryology.

Furthermore, I am very thankful to the BiOP team for huge amount of help in various microscopy-related questions, I am especially grateful to Olivier. Nicole and Tatiana did great jobs in steering me through the administrative mazes (that are, now I realize, nothing compared to the ones in Singapore…). Special thanks goes for my committee members, who agreed on allocating time for reading this thesis work and provided me useful comments on the projects: Anne Ephrussi, Joachim Lingner, Daniel Constam and Helge Grosshans.

I am extremely grateful for the support I received from my family. My mom always was/is/will be a strong pillar in my life, her presence, understanding, caring, curiosity, love helped me during these years. Unfortunately, my grandma, to whom this thesis work is dedicated cannot be here with us any more. I will never forget the moment when I was explaining her the processes of asymmetric cell division in C. elegans for a couple of hours drawing on sheets of papers. I am also thankful for the support and realistic criticism from my dad and for the enthusiasm of my sister. My sibling-cousins, Ádám, Eszter, Farkas and László and my uncle and aunt, Misi and Kinga, also contributed to create a stable basis of my life. I am immensely grateful for my girlfriend, Kalina, whose amazing personality and beautiful smile motivated and armored me a lot. I can thank a lot to my friends too. They might not even think, but they gave me a such personal energy that was necessary for completing my PhD. I am thankful to those who I had met before my arrival to Lausanne (impressive that almost all came to visit me): Hoki, Urbi, Era, Flóra, Peták, Dalma, Jals, Csöri, Norbi,
Halmos, Julian, Djoni, Matyi, Évi, Lőrinc, Kirschi, Áron, Szacsi, Tomi, Niki, Feri, Luca, Oszì, Marci, Gergő, Judit, Dani, Máté, Kinguc. And to those, who I met here: Vigorino, Lisalina, Tanjita, Livio, Rosy, Ilu, Fabri, Marco, Edu, Makuka úr, Szabesz, Gézu, Laci, Juan. Besides, numerous people had great influence on my life during these years, and I am grateful for all them for having made my life in Lausanne truly memorable.
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• C.R., Firnberg, N., Barres, B.A., Siderovski, D.P.


9. CURRICULUM VITAE

Personal Information
Date and place of birth: 1987, Budapest
Homepages:
http://www.linkedin.com/pub/zoltan-spiro/31/a5b/515
https://www.researchgate.net/profile/Zoltan_Spiro/

Education
- 2010 - Graduate studies at École Polytechnique Fédéral de Lausanne (EPFL)
- 2005 - 2010: Bachelor and Master studies at the Faculty of Biology, Eötvös Loránd University (ELTE), Budapest - specialized in Molecular Biology and Biochemistry

Research activity
- 2010 – onwards: graduate work at EPFL in the Institute of Cancer Research in the Gönöczy lab (gonczy-lab.epfl.ch)
- 2007-2010: laboratory work and Master project at Semmelweis University, Department of Medical Chemistry in the Soti lab (stressgroup.semmelweis-univ.hu)
- 2006-2007: undergraduate projects in the field of network biology (linkgroup.hu)

Publications – in preparation as of September, 2014
Spiró, Z. and Gönöczy, P: Asymmetric localization and MEX-5/6-dependent translational regulation of the novel mRNA *era-l* in C. elegans embryos
Spiró, Z. and Gönöczy, P: Centrosomal localization of *lin-5* mRNA contributes to asymmetric pulling forces during spindle positioning in C. elegans embryos

Publications – published as of September, 2014
links oxidative stress to the inhibition of heat stress adaptation', *Antioxidants & redox signaling* 17(6): 890-901.

**Conferences**
- 2011 September: Protein Synthesis and Translational Control, EMBL, Heidelberg
- 2012 September: The complex life of mRNA, EMBL, Heidelberg – poster presentation
- 2013 June: International Worm Meeting, UCLA, Los Angeles – poster presentation
- 2014 March: Basel Worm Meeting, FMI, Basel – oral presentation

**Scholarships**
- Scholar of the Hungarian Republic fellowship in 2008-2009 and in 2009-2010
- Scholar of Science fellowship of ELTE in 2008-2009 and in 2009-2010

**Science communication and social activities**
- Co-founder of the BioScience Network Lausanne (BSNL) ([www.bsnl.ch](http://www.bsnl.ch))
- Member of the organizing committee for EPFL-UNIL PhD retreats and SV Happy Hours
- Leader of Biology Students’ Association at Faculty of Biology at ELTE (2007-2010)

**Language skills**
- Hungarian (native), English (C1), German (A2), French (A2), Russian (A1)